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REMARKS

I. PROSECUTION HISTORY

In the outstanding Office Action mailed October 2, 2003, The Patent Office rejected claims 1-8, 10-17, 22-32, 49-58, and 63-100 on various grounds including 35 U.S.C. §§ 102 and 112, first paragraph. Claims 21, 33-48, and 59-62 were withdrawn from consideration. Claim 18 was allowed. Claims 1-8, 10-18, and 21-100 are currently pending.

II. RESPONSE TO CLAIM OBJECTIONS AND RESTRICTION REQUIREMENT

On page 2 of the Office action, the Patent Office maintained its revised (second) restriction requirement and made it final, and on page 3, the Patent Office objected to claims 22, 26-29, 49, 57-58, and 70-71 for allegedly containing non-elected subject matter, i.e., subject matter reciting VEGF-D. The Applicants respectfully traverse.

In response to the original restriction requirement in this case, in year 2000, the Applicants elected a Group drawn to a method of treating restenosis with a polynucleotide encoding a vascular endothelial growth factor. The elected group embraced use of either VEGF-C or VEGF-D polynucleotides. Three years later, on April 25, 2003, the Patent Office issued a further, narrower restriction, alleging that use of VEGF-C and VEGF-D polynucleotides were patentably distinct inventions. The Applicants elected, with traverse, Group I (claims 1-8, 10-18, 22-32, 49-74, and 75-100), drawn to a method of treating a mammalian subject to inhibit restenosis of a blood vessel by administering to the subject a composition comprising a polynucleotide that encodes a VEGF-C.

The Applicants did not, and do not, dispute that treatment with VEGF-C or VEGF-D represents patentably distinct inventions. Rather, the Applicants traversed on the ground that the MPEP indicates that two patentably distinct inventions should be restricted *only if examination together would pose a serious burden on the examiner.* The opposite situation exists here. For years, the Patent Office examined claims *in this application* whose scope included both VEGF-C and VEGF-D. See, Office actions of May 10, 2000, November 14, 2000, October 25, 2001, and June 5, 2002; Applicants' responses to each Office action; and Interview Summary of July 16, 2002. At this late stage in the prosecution, it would be inefficient to separate the examination into one directed to VEGF-C and another directed to VEGF-D, and there would be no serious burden to continuing to examine both groups of claims.

In the outstanding Office action, the Examiner asserted that a serious *search burden* would exist to examine both groups of claims, justifying a restriction. Even if that were initially the case, it is not the case with this RCE application, at this time, because searches have already been conducted on the full breadth of the restricted claims, during examination under the original restriction. For this reason, the Applicants request reconsideration and withdrawal of the new restriction requirement.

Because the restriction is still believed to be improper, the Applicants have not amended the claims at this time to remove VEGF-D subject matter.

III. THE SPECIFICATION AS FILED ENABLES THE CLAIMS

The Patent Office rejected claims 1-8, 10-17, 22-32, 49-58, and 63-100 under 35 U.S.C. § 112, first paragraph, alleging that the specification fails to enable practice of the invention using a nucleic acid that comprises "any portion or variant of amino acid sequences of SEQ ID NO: 2," or the use of "any viral or non viral vector." (Office action at pp. 3-6.) The Applicants respectfully traverse.

A. The Application As Filed Enables One Skilled In The Art To Practice The Claimed Invention With Polynucleotides That Encode Both VEGF-C And The Limited Genus Of Fragments And Variants Recited In The Claims.

The Patent Office acknowledged that the Applicants have enabled the use of polynucleotides that encode the complete human prepro-VEGF-C sequence set forth in SEQ ID NO: 2 to treat restenosis, and that VEGF-C binds to VEGFR-2 and VEGFR-3 and has been shown to stimulate both angiogenesis and formation of lymphatic blood vessels. (Office action at pp. 4-6.) Examples 1-4 of the specification represent working examples in which Applicants have shown that VEGF-C can be expressed in the aortic wall via gene therapy and reduce intimal thickening following aortic denudation, and have also shown the utility of VEGF-C gene transfer for preventing restenosis. *See, e.g.*, page 28, line 28, to page 29, line 2, and page 31, lines 19-36. Notwithstanding this evidence, the Patent Office alleged that "[t]he segments or variants of VEGF-C as claimed are mere hypothetical polypeptide[s] because no biological function has been established. (Office action at p. 6, lines 7-8.) The Applicants respectfully traverse.

1. The Claims Are Limited To Operative Embodiments.

At the outset, it is important to recognize that the claims do not encompass inoperative embodiments, as suggested in the Office action. Rather, the application identifies the VEGF-C of the claims to be molecules that retain VEGF-C activity. *See* specification at page 5, line 15, to page 7, line 7; and page 17, line 17, to page 19, line 21. Thus, the claims are properly tailored to read on operative embodiments.

2. The Scope Of VEGF-C Molecules Recited In The Claims Is Enabled By The Application.

The Patent Application contains an example where a gene therapy vector containing a full length "prepro-VEGF-C" cDNA sequence was used to inhibit restenosis. Based on what is known about the biology of VEGF-C, which is described in detail in the patent application,[1] a person skilled in the art would believe that this example is representative of the VEGF-C fragments recited in the current claim set.

The patent application explains that human prepro-VEGF-C polypeptide of 419 amino acids (SEQ ID NO: 2) is processed in multiple stages to produce *a mature and most active VEGF-C* polypeptide of about 21-23 kD (as assessed by SDS-PAGE under reducing conditions). Such processing includes cleavage of a signal peptide (SEQ ID NO: 2, residues **1-31**); cleavage of a carboxyl-terminal peptide (corresponding approximately to amino acids **228-419** of SEQ ID NO: 2 to produce a partially-processed form of about 29 kD; and cleavage (apparently extracellularly) of an amino-terminal peptide (corresponding approximately to amino acids **32-103** of SEQ ID NO: 2) to produced a fully-processed mature form of about 21-23 kD. Experimental evidence demonstrates that partially-processed forms of VEGF-C (*e.g.*, the 29 kD form) are able to bind the Flt4 (VEGFR-3) receptor, whereas high affinity binding to VEGFR-2 occurs only with the fully processed forms of VEGF-C. Moreover, it has been demonstrated that amino acids 103-227 of SEQ ID NO: 2 are not all critical for maintaining VEGF-C functions. A polypeptide consisting of amino acids **113-213** (and lacking residues 103-112 and 214-227) of SEQ ID NO: 2 retains the ability to bind and stimulate VEGF-C receptors, and it is expected that a polypeptide

[1] The second paragraph of the detailed description states, "The growth factor named Vascular Endothelial Growth Factor C (VEGF-C), as well as native human, non-human mammalian, and avian polynucleotide sequences encoding VEGF-C, and VEGF-C variants and analogs, have been described in detail in International Patent Application Number PCT/US98/01973, filed 02 February 1998 and published on 06 August 1998 as International Publication Number WO 98/33917; in Joukov *et al., J. Biol. Chem., 273(12)*: 6599-6602 (1998); and in Joukov *et al., EMBO J., 16(13)*: 3898-3911 (1997), all of which are incorporated herein by reference in the entirety. " The detailed description then goes on to summarize some of the important structure-function knowledge of VEGF-C biology.

spanning from about residue 131 to about residue 211 will retain VEGF-C biological activity. The cysteine residue at position 156 has been shown to be important for VEGFR-2 binding ability. However, VEGF-C ΔC_{156} polypeptides (*i.e.*, analogs that lack this cysteine due to deletion or substitution) remain potent activators of VEGFR-3. The cysteine at position 165 of SEQ ID NO: 2 is essential for binding either receptor, whereas analogs lacking the cysteines at positions 83 or 137 compete with native VEGF-C for binding with both receptors and stimulate both receptors.

The VEGF-C gene, protein, and variants have been the subject of multiple issued patents to Alitalo (one of the named inventors here) and Joukov. (*See, e.g.*, U.S. Patent Nos. 6,645,933; 6,403,088; 6,361,946; 6,221,839; 6,130,071. These patents, including their claims, embrace a number of biologically active VEGF-C forms other than the exact sequence used in the working example of this application.

Several important points should be apparent. First, segments and variants of VEGF-C are not mere hypothetical polypeptides whose function has not yet been established. Rather, the biology of VEGF-C has been characterized in detail, including the fact that more than 75% of the 419 amino acids of prepro-VEGF-C can be removed while retaining function. Second, the "activity" of VEGF-C actually *increases* when 100+ amino acids from the N-terminus and about 200 amino acids from the C-terminus are removed, because these "fully processed" polypeptides acquire VEGFR-2 signaling activity, whereas full length or partly processed forms signal through VEGFR-3 only. Third, the fact that the working example involved use of prepro-VEGF-C, and the fact that it is known that processing increases activity, provides a reason for one skilled in the art to expect that the invention will work with recombinantly processed (*e.g.*, truncated cDNA) forms of VEGF-C. Fourth, the VEGF-C activity assays, including those described in WO 98/33917 (incorporated in the present application by reference), enable one skilled in the art to identify VEGF-C variants that possess VEGF-C biological activity towards the known VEGF-C receptors (VEGFR-2 and VEGFR-3) and known target cell types. Fifth, the rejected claims define the VEGF-C molecule with structural and functional limitations that are consistent with the established biology of active VEGF-C and consistent with the scope of issued U.S. patent claims to VEGF-C molecules, *i.e.*, claims that the Patent Office has already agreed are enabled in scope. Finally, the Patent Office has failed to present any probative evidence to rebut the presumption of enablement. Evidence that other, unrelated proteins are affected by deletions

is certainly not relevant to the issue of enablement with respect to VEGF-C proteins, given the abundance of knowledge about VEGF-C that already exists.[2]

In conclusion, the biology of VEGF-C provides an unrebutted expectation that the claimed methods can be practiced effectively with the genus of VEGF-C molecules recited in the claims.[3]

B. The Application As Filed Enables One Skilled In The Art To Practice The Claimed Invention With Both Adenoviral Vectors, Other Viral Vectors, And Non-Viral Vectors As Well

From the bottom of page 4 to the bottom of page 5, the Patent Office alleges general limitations of gene therapy. However, this discussion does not address whether specific gene therapy regimens, *e.g.*, those of the present invention, are enabled. At best, the Patent Office establishes that gene therapy is not perfect and that it has limitations, but that is a description that would apply to any technology, and the failures of any technical field do not discredit the successes. As taught in the present application and subsequently filed declaration, and acknowledged by The Patent Office, anti-restenosis gene therapy with VEGF-C and -D was successfully achieved by Applicants. As the additional references discussed by the Applicants show, the Applicants' success is not an isolated success.

1. The Patent Office's Arguments And Cited References Regarding Gene Therapy Do Not Support A Conclusion Of Lack Of Enablement Of The Present Claims

That The Patent Office's description is not focused on the specific gene therapy methods claimed is revealed by such statements as "the problem to selectively target cells in vivo is still one of the most difficult obstacle[s] to overcome. The viral particles binds to many cells they encounter in vivo and therefor[e] would be diluted out before reaching their targets." That statement does not consider that the present application provides devices, materials, and methods to achieve *local* administration of gene therapy. The target cells for the present treatment are cells at or near the lumen of blood vessels, and materials

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[2] The Ngo article concerns itself with the search for a super algorithm that could be used to predict the tertiary structure of any protein from its primary sequence. The Rudinger article remarks that *a priori* that there is nothing to distinguish the sequences of peptide hormones from those of other peptides that lack function or have different function, but those peptide hormones are less than 10 residues long, and are not structurally related to VEGF-C.

[3] With respect to the withdrawn VEGF-D claims, it should be pointed out that VEGF-D biology also was known prior to the filing date, and that VEGF-D is processed in a manner similar to VEGF-C (at the N- and C-terminus), that VEGF-D and VEGF-C both bind VEGFR-3, and that fully processed VEGF-C and –D both bind VEGFR2. Thus, the arguments presented herein can also be made for VEGF-D claims.

and methods (*e.g.*, using catheters and coatings) have been developed to introduce gene therapy agents to these target cells locally. A number of the pending claims recite materials and methods for *local* delivery, and the examples show that local delivery works. Accordingly, dilution is not anywhere as great a concern with the present invention as with unrelated situations discussed by the Patent Office requiring systemic administration. The Patent Office also alleged difficulties in gene transfer to non-cycling cells. However, neointimal formations associated with restenosis do involve replicating cells because they involve pathogenic cell proliferation in a vessel. *See, e.g.*, page 2, lines 20-23. Moreover, this concern, too, is put to rest by the working example.

The Patent Office presents a number of review or popular interest articles regarding gene therapy. None of these articles includes a rigorous, systematic discussion of gene therapy that would be instructive for judging whether the present claims are enabled by the specification in view of those of skill in the art. Sweeping statements of how gene therapy is not as great as people hoped it would be does not support a lack of enablement of the present specification, which provides specific examples that adequately allow one of skill in the art to practice the claimed invention. That gene therapy has not been perfected does not mean that the present disclosure is not enabling.

Much of what is cited by The Patent Office pertains to regulatory matters that are the province of the FDA or other governmental entities that have different standards and considerations, and which are not determinative of enablement: "considerations made by the FDA for approving clinical trials are different from those made by the PTO in determining whether a claim is enabled." M.P.E.P. § 2164.05. Much of the general gene therapy literature that The Patent Office cites was also written in the wake of the tragedy of a single gene therapy patient undergoing therapy directed to a metabolic enzyme deficiency, *see* beginning of the Friedmann article. This tragedy forced a rethinking of the regulation of gene therapy, but it did not impinge on issues of enablement.

After the heading "State of Art and Predictability" on page 4 of The Patent Office Action, The Patent Office states that "gene therapy is considered [a] highly experimental area of research at this time." Medical research by definition has involved and continues to involve considerable experimentation; gene therapy is not unique in this respect, and it does not imply "undue experimentation," the relevant criterion, among others, in evaluating the present claims for enablement. It is also not clear what "time" The Patent Office is referring to as the articles cited to support The Patent Office's broad statement were

written over a period of approximately five years, and are concerned an even longer period of time. The Touchette article sent by The Patent Office is dated January 1996, over two years before the present application's priority date.

The Patent Office's statement continues: "researchers and the public agree that demonstrable progress to date has fallen short of initial expectations." What the public "expects" is not relevant to the evaluation of enablement, especially since the benchmark level of "expectation" has not been established for evaluation here. Nor is the ambiguous statement about "initial expectations." Disappointment as to the progress of a technology is not a relevant criterion of enablement, or even a meaningful statement about the level of progress achieved.[4]

The Patent Office's statement towards the bottom of page 5 regarding gene therapy protocols being "oversold" is also irrelevant to enablement. Technologies are often "oversold," that is nothing unique to gene therapy, and hyping of a technology does not undermine its actual strengths and successes. Past failures with other genes to treat other types of conditions do not discredit successes that have been made, *e.g.*, that described in the present application.

Apart from the article that includes the subject matter, and authored in part by the Applicants, of the present application, the DeYoung and Dichek article is the one article cited by The Patent Office that focuses on gene therapy in the context of restenosis. That article was written before the filing of the present application and the publication of the Applicant's journal article. Had DeYoung and Dichek known about Applicants research they very well may have come to a more positive assessment regarding the use of gene therapy to treat restenosis.

The Patent Office states that "the pathophysiology of restenosis is incompletely understood," but that is a statement that would apply to any disease. Similarly, that "[n]o cures can as yet be attributed to gene therapy" does not discredit gene therapy as a treatment that ameliorates certain conditions even if a complete cure is not obtained. The present claims are directed to methods of treatment, there is no requirement that restenosis be cured or prevented altogether.

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[4] Legions of cancer patients and other types of patients are "disappointed" when patented chemotherapeutic drugs or other materials and methods for therapy do not cure their cancers or other conditions.

The Patent Office fails to note that its cited references also include a number of positive statements regarding gene therapy. For example, the Friedman article, top of col. 3, page 2163, "Adverse Results Do Not Invalidate the Rationale for Gene Therapy." And in the Verma article, lines 18-19: "We hope that these new guidelines will be sensible and practical and not adversely affect the *current steady progress in the field of gene therapy.*" (Emphasis added.) This passage shows not only the positive direction of gene therapy, but also indicates that the problem at the time was not of the effectiveness of gene therapy, but rather a concern for working out proper regulations. The fact that the Verma article actually starts out with a statement regarding "[s]uccess in gene therapy" is particularly supportive of gene therapy as a technique that works.

2. The Application Provides A Working Example That Is Representative And Enabling Of The Claims As A Whole

The Patent Office alleged that Applicants had only demonstrated that an adenovirus could be used for restenosis VEGF-C gene therapy, and that the use of other viral or non-viral vectors had not been enabled. As discussed above in section III. A. 1, Applicants have provided a number of working examples in the specification. The present application also teaches that: "Any suitable vector may be used to introduce the VEGF-C transgene into the host." Specification at page 8, lines 23-24. The specification lists the different vectors that may be used including both viral and non-viral and citing relevant literature for each, page 8, line 24, to page 9, line 3. This information and that described elsewhere in the specification, *e.g.*, in the examples, is sufficient to enable those of skill in the art to practice the claimed invention. "The evidence provided by applicant need not be conclusive but merely convincing to one skilled in the art." M.P.E.P. § 2164.05 (underlining in original). Applicants have meet that burden.

3. Published Studies Demonstrate That The Specification Is Enabling

"As long as the specification discloses at least one method for making and using the claimed invention that bears a reasonable correlation to the entire scope of the claim, then the enablement requirement of 35 U.S.C. 112 is satisfied." M.P.E.P. § 2164.08 "An applicant need not have actually reduced the invention to practice prior to filing." M.P.E.P. § 2164.02. Data published subsequent to the filing of the present application demonstrates that the invention described in the specification as filed can be practiced successfully.

a. Use of Naked DNA As the Vector

Example 5 provides a protocol for treating restenosis using a protocol similar to that which is explained in Example 1 of the specification except that instead of using an adenovirus with VEGF-C encoding DNA, "naked" VEGF-C plasmid DNA is used. Yoon, *et al., J. Clin. Investig. 111(5):* 717-725 (2003) (hereinafter "Yoon," Exhibit A), demonstrates that a naked VEGF-C DNA protocol can be effective *in vivo* (rabbit model) for modulating a biological system. Yoon's Figure 3 at page 720 demonstrates successful gene transfer of naked VEGF-C DNA plasmid. Moreover, Yoon is not the only example of naked VEGF-C DNA gene therapy. Corautus Genetics is conducting clinical trials using naked VEGF-2 DNA plasmid therapy[5]--VEGF-2 is a molecule with approximately 99% homology to full length VEGF-C at the amino acid level. Thus, there is evidence that VEGF-C gene therapy can be effective even *without* a vector in the traditional sense with which that term is used in gene therapy. Of course, vectors that lead to increased expression, such as the adenoviral vector used by the applicants in their example and other vectors identified in the application, can be expected to be even more effective. Collectively, these data refute the Patent Office's allegations that one would not predict success when extending the Applicant's working example with other vectors.

b. Use Of Other Types Of Vectors: Successful Transfer Of Other Vascular Endothelial Growth Factor Genes By Both Adenoviral And Other Methods Demonstrate The Enabling Disclosure Of The Present Application

Success of the present invention involving VEGF-C (or –D) gene therapy with multiple vectors also would be predicted from successes with other gene transfers. For example, VEGF-A (VEGF, VEGF165), another vascular endothelial growth factor, has been transferred by adenovirus. See Gene Therap. 9(19):1271-7 (Exhibit B). In addition, VEGF-A has also been successfully transferred using other vectors.

For example, Bellomo and co-workers reported the successful transfer of VEGF-A using an adeno-associated virus, which as mentioned above has also been successfully transferred using adenovirus. Pharmacological Res. 48:309-317 (2003) (Exhibit C). Use of adeno-associated vectors for introduction of the VEGF-C transgene is described at page 8, line 27, of the specification.

[5] Relevant pages from the company's website, including discussion of Phase II trials, are provided in Exhibit F. Exhibit F also includes an article (Fortuin, *et al., Am. J. Cardiol.*, 92:436-439 (2003)) regarding a phase I study of VEGF-2 naked plasmid DNA gene therapy. That study was supported by Vascular Genetics, a predecessor company to Corautus Genetics.

Pels and co-workers, including one of the present Applicants (Seppo Yla-Herttuala), reported successful transfer of VEGF-A using liposomes and lipofectin to affect vascular thickening in the context of coronary artery balloon injury, see abstract and at page 665 2nd col., last paragraph. Cardiovascular Res. 60:664-72 (2003) (Exhibit D). Use of liposomal vectors/lipofectin-mediated gene transfer for the introduction of the VEGF-C transgene is described at page 8, lines 30-31, of the specification.

Alian and coworkers report the successful transfer of VEGF-A using both adenoviral and retroviral transfer, see abstract and at page 3, col. 1. J. Virological Meth. 105:1-11 (2002) (Exhibit E). Use of retroviral vectors for the introduction of the VEGF-C transgene is described at page 8, lines 24-26.

These references pertaining to VEGF-A gene therapy with multiple vectors are certainly more relevant to the claimed invention that the generic literature cited by the Patent Office. These published studies collectively support a conclusion that the specification, as filed, properly enabled gene transfer of a polynucleotide encoding VEGF-C polypeptides with multiple vectors, and that persons of ordinary skill would have reason to expect that success demonstrated in the application with an adenovirus vector could be extended with other vectors as well.

For the reasons discussed above, the specification enables the full scope of the claims, and would allow anyone of skill in the art to transfer any VEGF-C polypeptide encoding polynucleotide using any vector to treat restenosis in the context of the claimed method. Accordingly the rejection is improper and should be withdrawn.

IV. WO97/05250 DOES NOT ANTICIPATE THE CLAIMS BECAUSE THE REFERENCE FAILS TO DISCLOSE EACH AND EVERY ELEMENT OF THE CLAIMS AND BECAUSE THE LABEL IS FUNCTIONALLY RELATED TO THE CONTENTS OF THE CLAIMED KIT

The Patent Office maintained the rejection of claims 29 and 71-72 under 35 U.S.C. § 102(b) as allegedly anticipated by Alitalo WO/97/05250 (hereinafter '250), and extending the rejection to claims 98 and 100. The applicants respectfully traverse for the reasons given previously and for the reasons as follow.

A. WO 97/05250 Does Not Anticipate The Claims Because The Reference Fails To Disclose Each And Every Element Of The Claims

"A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." M.P.E.P. § 2131.

"[W]hen evaluating the scope of a claim, every limitation in the claim must be considered. Office personnel may not dissect a claimed invention into discrete elements and then evaluate the elements in isolation. Instead, the claim as a whole must be considered." M.P.E.P. § 2106 (underlining in original).

As Applicants pointed out in their response to the June 5, 2002 Office action, the Federal Circuit in *In re Gulack* echoed the M.P.E.P. language: "The Board cannot dissect a claim, excise the printed matter from it and declare the remaining portion of the mutilated claim to be unpatentable." 703 F.2d 1381, 1385; 217 U.S.P.Q. 401 (Fed. Cir. 1983).

Claim 29, for example, recites not only VEGF-C polynucleotides but also a container and "a label attached to or packaged with the container, the label describing use of the agent for inhibition of restenosis of a blood vessel. '250 does not recite such a label, accordingly '250 cannot anticipate the claims. Accordingly, the rejection is improper and the rejection should be withdrawn.

B. The Label Is Functionally Related To The Contents Of The Claimed Kit

The Patent Office cites *Gulack*: "Where the printed matter is not functionally related to the substrate, the printed matter will not distinguish the invention from the prior art in terms of patentability." 703 F.2d 1381, 1385. The key phrase is "functionally related." The *Gulack* Court explained this concept further by citing *In re Miller* (418 F.2d 1392, 163 USPQ 46 (CCPA 1969). In *Miller*, the rejection had been based on a lack of structural relationship, but the *Miller* Court rejected that reasoning: "[i]t seems to us that what is significant here is not structural but *functional* relationship." 703 F.2d 1381, 1385-86 (emphasis in the original).

The proper legal analysis is to examine the functional relationship between the printed matter and the other subject matter of the claim. However, The Patent Office instead focuses on the structural relationship, and distinguishes the claimed subject matter in *Gulack* from the present claims on the basis that in *Gulack* the printed matter was physically located on a band, whereas the claimed label is not written out in the genetic code. This structural

relationship and distinction is irrelevant and does not address the correct legal standard of functionality as discussed above.

The Patent Office also asserted that the label and the nucleic acids of the kit are not related in any functional way. This assertion completely ignores the fact that the printed matter in claim 29 does recite a novel and nonobvious functional relationship in that it directs a medical application for the polynucleotides that was never suggested before. There is no prior art suggesting the use of VEGF-C agents to inhibit restenosis, which means that present kit claims can also not be anticipated.

Moreover, the standard as stated above is a functional **relationship** between the printed matter and substrate, not the function of the nucleic acids of the kit as asserted by The Patent Office or even the overlying invention--which is the entire kit, not just contents of the kit, *i.e.*, the nucleic acids. The functional relationship in the present kit claims is that the label tells one how to administer the contents of the kit to treat restenosis. Again, in examining the claims, The Patent Office must consider all elements of each claim. Because The Patent Office has failed to do so, the rejection is improper and should be withdrawn.

C. There Is Patent Office Precedent For Claims Of The Form Presented By The Applicants

The Patent Office has previously issued claims of the type presented by the current applicants. *See, e.g.*, Claim 29 of U.S. Patent No. 6,598,603 ("29. A kit for treating respiratory diseases, the kit comprising (a) a budesonide composition in a sealed **container**, the composition containing 0.05 mg to 15 mg budesonide and a solvent, and (b) a **label** indicating administration by nebulization in a continuing regimen at a frequency of not more than once per day.); claim 15 of U.S. Patent No. 6,475,796("15. An article of manufacture comprising a VEGF variant ...; a **container; and a label** or package insert comprising instructions for administration of said VEGF variant."); claim 40 from U.S. Patent No. 6,432,934 ("40. A transdermal delivery kit, comprising a delivery device, and in a sealed **container** the formulation of claim 38, and **instructions** for its administration."). Thus, there is recent precedent for exactly the type of claim presented by the Applicants.

In their response to the June 5, 2002 Office action, Applicants also discussed claims of recently issued patents that claimed kits using language analogous to the present claims. The Patent Office has failed to respond to that discussion, and Applicants respectfully request consideration and comment on the same by The Patent Office.

D. Summary

The '250 reference does not anticipate the claims because the reference fails to disclose each and every element of the claims and because the label is functionally related to the contents of the claimed kit. Moreover, the kit claims as presented are in an acceptable format as recognized by The Patent Office. For all of these reasons the rejection is improper and should be withdrawn.

CONCLUSION

The applicants respectfully request prompt reconsideration of the pending claims. The claims are believed to be in condition for allowance in view of the foregoing amendments and remarks. Withdrawal of the rejections and allowance of the claims are respectfully solicited.

While a two month extension fee is due, no further fees are believed due. The Commissioner is hereby authorized to charge the one month extension fee and any other fees due with this response to Deposit Account No. 13-2855.

The examiner is invited to contact the undersigned at the telephone number listed below in order to discuss any remaining issues or matters of form that will move this case to allowance.

Respectfully submitted,

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March 2, 2004

VEGF-C gene therapy augments postnatal lymphangiogenesis and ameliorates secondary lymphedema

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Although lymphedema is a common clinical condition, treatment for this disabling condition remains limited and largely ineffective. Recently, it has been reported that overexpression of VEGF-C correlates with increased lymphatic vessel growth (lymphangiogenesis). However, the effect of VEGF-C–induced lymphangiogenesis on lymphedema has yet to be demonstrated. Here we investigated the impact of local transfer of naked plasmid DNA encoding human VEGF-C (phVEGF-C) on two animal models of lymphedema: one in the rabbit ear and the other in the mouse tail. In a rabbit model, following local phVEGF-C gene transfer, VEGFR-3 expression was significantly increased. This gene transfer led to a decrease in thickness and volume of lymphedema, improvement of lymphatic function demonstrated by serial lymphoscintigraphy, and finally, attenuation of the fibrofatty changes of the skin, the final consequences of lymphedema. The favorable effect of phVEGF-C on lymphedema was reconfirmed in a mouse tail model. Immunohistochemical analysis using lymphatic-specific markers: VEGFR-3, lymphatic endothelial hyaluronan receptor-1, together with the proliferation marker Ki-67 Ab revealed that phVEGF-C transfection potently induced new lymphatic vessel growth. This study, we believe for the first time, documents that gene transfer of phVEGF-C resolves lymphedema through direct augmentation of lymphangiogenesis. This novel therapeutic strategy may merit clinical investigation in patients with lymphedema.

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Introduction

Lymphedema is defined as the progressive accumulation of protein-rich fluid in the interstitial spaces that results from an anatomic or functional obstruction in the lymphatic system (1). While primary lymphedema occurs infrequently on a hereditary or idiopathic basis, secondary lymphedema is common worldwide, primarily due to the increase in radical surgery and radiotherapy for cancer in developed countries and infectious disease (filariasis) in developing countries (2, 3). Despite substantial advances in both surgical and conservative techniques, therapeutic options for management of lymphedema are limited (3, 4). Pathophysiologically, restoration of the lymph-transporting capacity would appear to represent the optimal treatment for lymphedema. However, no means for accomplishing new lymphatic channel development currently exists. Growth of new lymphatic vessels (lymphangiogenesis) in healthy animals is rapid. The best example of the natural recovery of lymphatic drainage in animals is the complete restoration of lymphatic flow after limb reimplantation (5–9). Therefore the primary difficulty found in lymphedema animal models is to develop a method to sustain lymphedema long enough to allow evaluation of therapies.

Recent molecular studies have begun to elucidate the basis for lymphangiogenesis that can be stimulated by various cytokines, including VEGF-C (VEGF-2) (10, 11). VEGF-C, the first ligand to be discovered for VEGFR-3 (Flt4), is a member of the VEGF family of polypeptide growth factors. VEGF-C binds to endothelial cell receptors VEGFR-2 (Flk1) and VEGFR-3 (12–15). Although VEGFR-3 plays a critical role for both vascular and lymphatic endothelial cell develop-

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Nonstandard abbreviations used: naked plasmid DNA encoding human VEGF-C (phVEGF-C); coefficient of variation (cv); lymphatic endothelial hyaluronan receptor-1 (LYVE-1); platelet endothelial cell adhesion molecule-1 (PECAM-1).

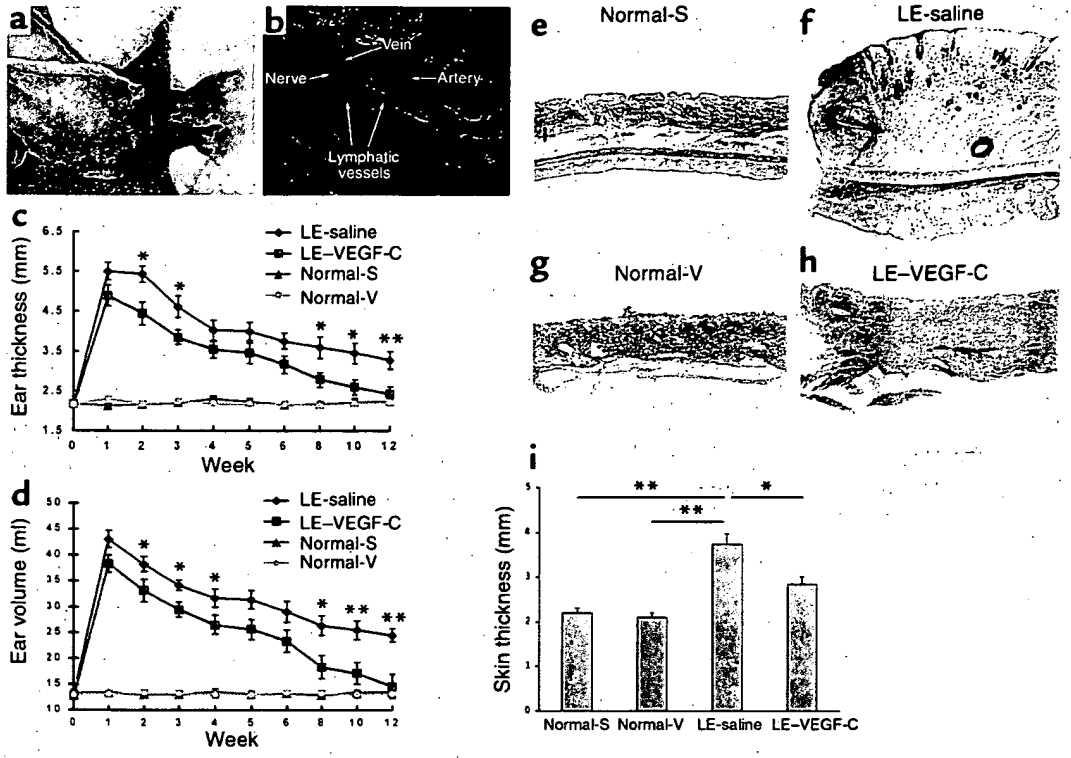

Figure 1

(**a–d**) Rabbit ear model of lymphedema: effect of phVEGF-C gene therapy. (**a**) Postoperative appearance of the dorsal surface of the rabbit ear. Lymphedema surgery leaves a gap of cartilage crossed only by the skin bridge. (**b**) A view under a surgical microscope after lifting up the skin bridge showing neurovascular bundle. Lymphatic vessels were visualized as blue lines (arrows) due to the uptake of Evans blue. Ear thickness (**c**) and volume (**d**) show consistent differences between the VEGF-C and saline groups over 12 weeks. $*P < 0.05$; $**P < 0.01$. (**e–i**) Decreased skin thickness after phVEGF-C transfer in a rabbit lymphedema model. Photos show cross sections of the skin after elastic-tissue trichrome staining 8 weeks after lymphedema surgery. Compared with normal ears (**e** and **g**), operated ears (**f** and **h**) had fibrofatty tissue deposition and thus greater skin thickness. The phVEGF-C–transfected ear shown in **h** shows less fibrosis and decreased thickness compared with the saline-injected ear (**f**), which demonstrates other characteristic features of lymphedema, such as profound epidermal hyperplasia and papillomatosis. (**i**) Measurement of skin thickness from histologic sections shows a significant difference between the saline and VEGF-C groups ($P < 0.05$). $*P < 0.05$; $**P < 0.01$. Scale bar, 500 μm. Normal-S and Normal-V indicate unoperated ears from the saline and VEGF-C groups, respectively; LE indicates lymphedema-operated ears.

ment, its expression becomes limited to the lymphatic endothelium beginning in the late stages of development (16–18). Overexpression of VEGF-C cDNA in the skin of transgenic mice induced lymphatic endothelial cell proliferation and hyperplasia of the lymphatic vasculature, and recombinant VEGF-C specifically stimulated lymphangiogenesis in chorioallantoic membrane (11, 19). Recently, direct evidence of the link between VEGFR-3 and lymphedema has been found: it has been reported that human hereditary lymphedema is associated with heterozygous missense mutation of the *Flt4* gene, which leads to insufficient VEGFR-3 signaling (20, 21). Recently, it was demonstrated that subcutaneous injection of adenovirus or adeno-associated virus encoding VEGF-C could generate lymphatic vessels in the skin of normal mice (22) and in a mouse model (*chy* mouse) of primary lymphedema (23). Although these studies showed that VEGF-C could induce lymphangiogenesis in vivo, they failed to show that this VEGF-C–induced lymphangiogenesis could improve overall lymphatic vascular dysfunction and prevent chronic changes accompanied by lymphedema, which are the key determinants of whether VEGF-C can be used as a therapeutic option to treat human lymphedema.

Accordingly, first we sought to establish reliable animal models of secondary lymphedema. The two animal models used here provided complementary measurements: the rabbit ear had the advantage of size, which is conducive to direct measurements and lymphoscintigraphy, whereas the mouse tail had advantages for immunohistochemistry owing to the availability of lymphatic vessel–specific Ab's. Next, using these animal models, we investigated whether local transfer of naked plasmid DNA encoding human VEGF-C (phVEGF-C) could promote lymphangiogenesis and improve physical, functional, and pathologic aspects of lymphedema.

Methods

All animal protocols were approved by the Institutional Animal Care and Use Committee of St. Elizabeth's Medical Center. Investigators for the follow-up examinations were blinded to the identity of the treatment given.

Rabbit ear model of lymphedema. We modified several previous rabbit ear models to overcome the shortcomings of rapid lymphatic regeneration and to provide the bed for new lymphatic vessel growth (5, 24, 25). To meet those requirements, we used old (3–4 years of age) New Zealand White rabbits and created a skin bridge. Before the operation, the lymphatic vessels were identified by intradermal injection of 0.2 ml of 1% Evans blue dye at the dorsal tip of the right ear. A strip of skin, subcutaneous tissues, and perichondrium 3 cm wide was circumferentially excised from the base of the ear, except for the central portion (1 cm in width) of the dorsal skin, i.e., a "skin bridge" underneath which runs the neurovascular bundle (Figure 1b). After the distal edge of the skin bridge was incised, lymphatic channels were dissected and the lymphatic stumps were resected under a dissecting microscope. Other edges of skin were inversely sutured to the perichondrium to prevent reapproximation of skin edges and recanalization of the lymphatic vessels. This created a strip of bare cartilage, leaving only the skin bridge for lymphatic growth (Figure 1a).

Preparation of phVEGF-C and gene transfer protocol in a rabbit model. A total of 54 rabbits was randomized into two groups in a blinded fashion for treatment with phVEGF-C or control (saline). In the VEGF-C group, 500 μg of phVEGF-C in 0.5 ml volume was injected intradermally and subcutaneously at the skin bridge using a 27-gauge needle on days 1, 6, and 11 after lymphedema surgery.

Measurement of ear thickness and volume. The thickness of the rabbit ears was measured 1 cm medial and distal to the medial border of the skin bridge with a vernier caliper. The ear was put in a 50-ml cylinder filled with water. After removing the ear, the volume of water displaced by the ear was measured (25). The thickness and volume of all ears was measured before surgery and every week for 6 weeks, and thereafter every 2 weeks until the 12-week point (n = 12 in each group).

Measurement of skin thickness in histologic sections. Thickness of the ear skin at 8 weeks after surgery was measured in a cross section of the skin just distal to the skin bridge in paraffin-embedded histologic specimens after elastic-tissue trichrome staining as described previously (26) n = 5 in each group).

Lymphoscintigraphy and quantitative analysis. Tc-99m-filtered sulfur colloid was injected intradermally into the dorsal tip of rabbit ears at a dose of 50 μCi. Imaging was performed using a large-field-of-view Genesys γ camera (ADAC Laboratories, Milpitas, California, USA).

To quantitatively compare lymphatic drainage of the injected radiotracers, radioactivity within the ears was counted. The γ counts at injection sites were similar in both ears of the saline and VEGF-C groups (P = 0.93). For standardization, the ratio of radioactivity of the operated ear to that of the normal (contralateral) ear, designated the radioactivity index, was used to compare lymphatic drainage at 4, 8, and 12 weeks (see Figure 2, g–i). The validity of the radioactivity index was verified by repeated examination of normal ears (n = 7) for intraindividual variation, which was 6% (coefficient of variation [cv %]), and by comparison of the day 1 postoperative lymphoscintigrams for interindividual variation (n = 20), which was 10% (cv %).

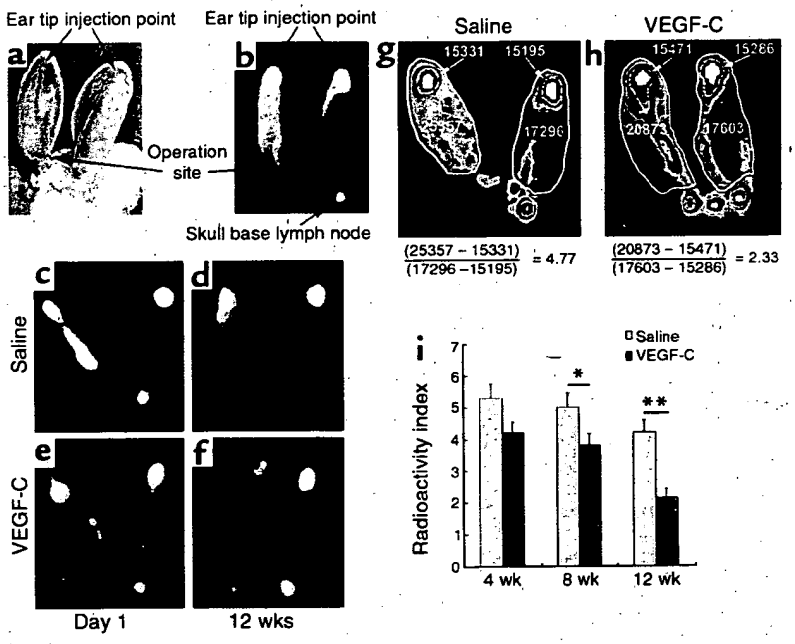

Figure 2

Temporal changes of lymphatic function visualized by lymphoscintigraphy. (**a** and **b**) Orientation of the lymphoscintigraphic images. In normal ears, lymphatic flow assumes a linear pattern and the draining LNs are clearly visible. In the operated ear, the lymphatic passages were blocked, resulting in backward diffusion and no visualization of LNs. (**c** and **d**) Temporal changes in the saline group. Even at 12 weeks (**d**), lymphoscintigraphy demonstrates substantial impairment of lymphatic drainage of the saline-injected ear, indicated by dermal backflow and faint visualization of the LNs. (**e** and **f**) Temporal changes in the VEGF-C group. In the phVEGF-C–transfected ears, there was remarkable improvement of draining function. At 12 weeks, a linear passage of radiotracer, decreased dermal backflow, and increased uptake by LNs were observed. (**g** and **h**) Representative lymphoscintigraphic images and calculation of radioactivity index from the saline (**g**) and VEGF-C group (**h**). To quantitatively compare lymphatic drainage, the radioactivity within the ear was counted. Net radioactivity of the ear was obtained by subtracting γ counts at injection sites (arrows) from the total counts of the ear. The radioactivity index is the ratio of radioactivity of the operated ear divided by the radioactivity of the normal ear; this was used to compare lymphatic drainage function of the lymphedema ears. Higher ratios indicate more persistent radioactivity and less lymphatic drainage. (**i**) Comparison between the saline and VEGF-C groups shows the values were consistently lower in the VEGF-C group at 4, 8, and 12 weeks. $^*P < 0.05$; $^{**}P < 0.01$.

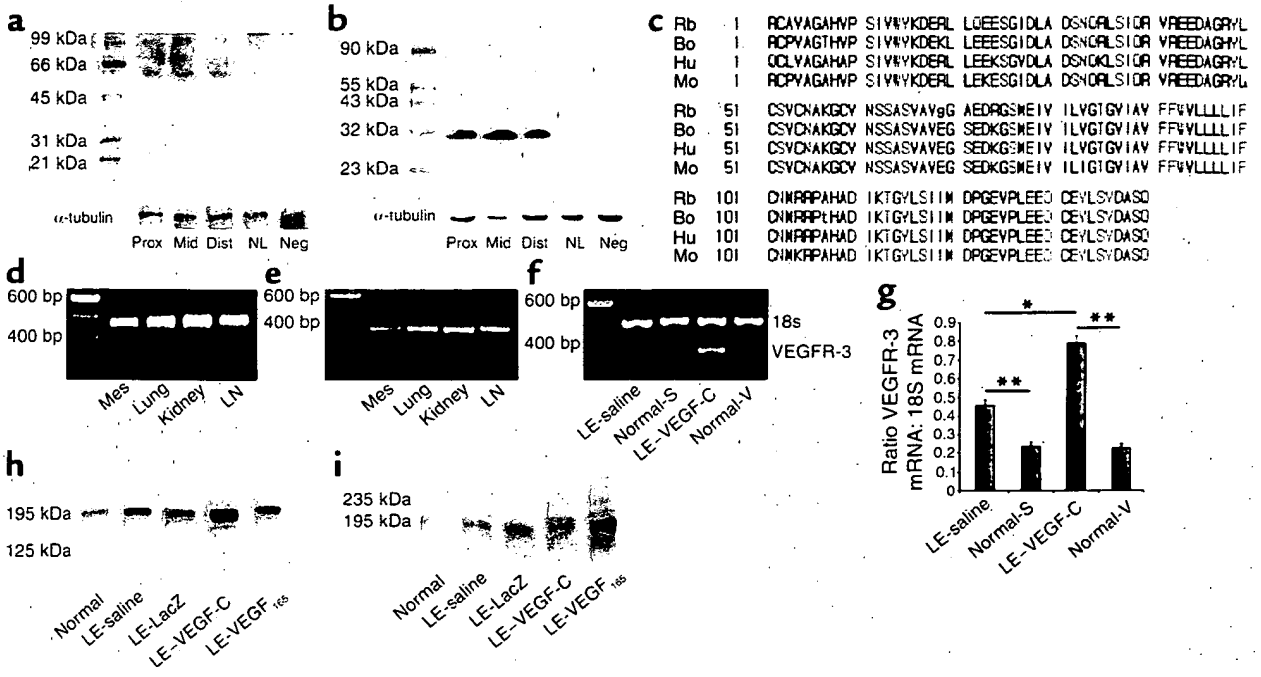

Figure 3

Increased expression of VEGF-C protein and VEGFR-3 mRNA in the phVEGF-C–transfected ears. (**a** and **b**) Western blot of VEGF-C protein from skin. VEGF-C was detected in its 58-kDa (**a**) and 31-kDa forms (**b**). VEGF-C protein expression was significantly higher at and around the phVEGF-C–transfected lymphedema skin. Prox, Mid, and Dist represent samples obtained from ear skin proximal to the skin bridge, skin from the bridge itself, and intact ear skin just distal to the skin bridge of the phVEGF-C–transfected ear, respectively. Neg, samples from the skin bridge of saline-injected lymphedema ear. NL, samples from the bridge site of unoperated contralateral ear. (**c** and **d**) Using degenerate oligonucleotides, RT-PCR was performed for total RNA extracted from mesentery (Mes), lung, kidney, and LNs. The PCR product (470 bp) from the kidney sample was sequenced. At the protein level, the rabbit (Rb) VEGFR-3 clone displayed 92.9%, 93.6%, and 94.3% identity with human (Hu), bovine (Bo), and mouse (Mo) VEGFR-3, respectively. (**e**) New primer sets were designed from the sequenced rabbit VEGFR-3 DNA, yielding a single PCR product of 362 bp. (**f**) Representative semiquantitative RT-PCR showing higher expression of VEGFR-3 in the lymphedema skin transfected with phVEGF-C than in the saline-injected or unoperated skin. (**g**) Quantification of VEGFR-3 mRNA levels. (*$P < 0.001$. **$P < 0.01$). (**h** and **i**) The effect of phVEGF-C gene transfer on tyrosyl phosphorylation of VEGFR-3 (**h**) and VEGFR-2 (**i**) by immunoprecipitation with anti-phosphotyrosine Ab followed by Western blot analysis with anti–VEGFR-3 or anti–VEGFR-2 Ab's, respectively. Samples transfected with phVEGF-C revealed similar levels of phosphorylated VEGFR-2 compared with the control groups (saline and LacZ).

Western analysis of VEGF-C transgene expression in tissue. Samples harvested from the skin bridge and from tissue proximal and distal to the skin bridge of the operated ears, and from the bridge site of the contralateral ears were snap frozen in liquid nitrogen 7 days after the second injection of phVEGF-C (postoperative day 13) (n = 5 in each group). Western analysis was performed as described (27).

Molecular cloning of partial rabbit VEGFR-3 cDNA. Because the rabbit VEGFR-3 DNA sequence has not been identified, we sequenced part of the VEGFR-3 cDNA using degenerate oligonucleotides. Degenerate oligonucleotides were designed from conserved amino acid sequences NVSDSLEM and WEFPRER, located at the transmembrane domain of human and mouse VEGFR-3 (28, 29). The deduced oligonucleotide sequences were 5′-AACGTGAG (CT)GACTC (GC)(CT)T (AGCT)GA (AG)ATG-3′ and 5′-CC (GT)YTC (CT)C (GT)GGG (AG)AA (CT)TCCCA-3′, respectively. A single PCR product of 470 bp was obtained from all the tissues (see Figure 3d).

Semiquantitative RT-PCR analysis of VEGFR-3. Using samples harvested from the bridge site of both ears at postoperative day 13 (n = 5 in each group), total RNA was isolated and RT-PCR was performed as described above. The primer pair used, designed on the basis of the sequenced cDNA's for rabbit VEGFR-3, was 5′-TATGGTACAAAGATGAGAGGC-3′ (sense) and 5′-ACAGGTATTCACATTGCTCCT-3′ (antisense). To quantify the VEGFR-3 mRNA, we used the "competimer" quantitative PCR technique (Ambion Inc., Austin, Texas, USA) according to the manufacturer's instructions. To the VEGFR-3 PCR mix, we added a mix of 18S primer pairs and 18S 3′-end modified primers (competimers) at a ratio of 1:9, yielding a 488-bp product. PCR was performed as follows: 94°C, 2 min (once); 94°C, 15 s; 50°C, 30 s; 72°C, 1 min (40 cycles); 72°C, 10 min (once). PCR products were separated on 1.5% agarose gel and quantified by integrated density analysis software (EagleSight Software 3.2; Stratagene, La Jolla, California, USA).

Mouse tail model of lymphedema. Male nude (*nu/nu*) mice (Harlan, Indianapolis, Indiana, USA) 12 weeks of

age were used. A mouse lymphedema model was created by modifying a previous model (30).

Gene transfer protocol in a mouse tail model. In total, 115 mice were randomized into five groups: no operation, VEGF-C, $VEGF_{165}$, LacZ, and saline (n = 23 in each group). The unoperated group served as negative control. The other groups underwent the operation as described. In the VEGF-C group, 100 μg of phVEGF-C was given in 100 μl volume on days 1, 6, and 11 after the operation, respectively. The $phVEGF_{165}$ plasmid (18, 31), pGSV-nlsLacZ (32) (a nuclear targeted LacZ gene plasmid encoding the protein β-galactosidase), and saline were injected in an identical fashion in the $VEGF_{165}$, LacZ, and saline groups, respectively.

Immunoprecipitation of receptor phosphorylation. To investigate the effect of VEGF-C overexpression on phosphorylation of VEGFR-2 and VEGFR-3, immunoprecipitation and Western blot analysis was performed in the mouse tail model. Lysis of tissues, immunoprecipitation, and Western blot analysis were performed as described (31, 33). Aliquots of protein extracts (1 mg) were incubated for 2 hours at 4°C with 3 μg of mAb against phosphotyrosine (Upstate Biotechnology Inc., Lake Placid, New York, USA), followed by incubation with 40 μl of protein G–agarose beads (Roche Diagnostics GmbH, Mannheim, Germany) overnight at 4°C. Immunoprecipitates of tyrosine-phosphorylated proteins were separated by 7.5% SDS-PAGE and electrotransferred onto PVDF membranes. The membranes were immunoblotted overnight at 4°C with a rabbit polyclonal Ab against VEGFR-3 (1:500; Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) or VEGFR-2 (1:500; Santa Cruz Biotechnology Inc.).

Immunohistochemistry and morphometric analysis. The skin from the bridge area was harvested 3 weeks after plasmid/saline injections. Skin sections were stained using a rat mAb against mouse VEGFR-3 (34) and a rabbit polyclonal Ab against the lymphatic marker lymphatic endothelial hyaluronan receptor-1 (LYVE-1), a receptor for hyaluronan and a homologue to the CD44 glycoprotein (35).

In double fluorescent immunohistochemistry of LYVE-1 and Ki-67, LYVE-1 staining was performed with the use of Texas red–streptavidin (NEN Life Science Products Inc., Boston, Massachusetts, USA), and Ki-67 staining was performed with rabbit polyclonal Ab against Ki-67 (Novocastra Laboratories Ltd., Newcastle, United Kingdom) and Cy2-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania, USA). Endothelial cells were identified by immunohistochemical staining for platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31) with a rat mAb against mouse CD31 (BD Biosciences, San Diego, California, USA) (36) in mouse tissues and with a mouse mAb against human CD31 in rabbit tissues.

Statistical analysis. All results were expressed as mean ± SEM. Statistical analysis was performed with an unpaired Student t test for comparisons between two groups and ANOVA followed by Scheffe's procedure for more than two groups. P values < 0.05 were considered to denote statistical significance.

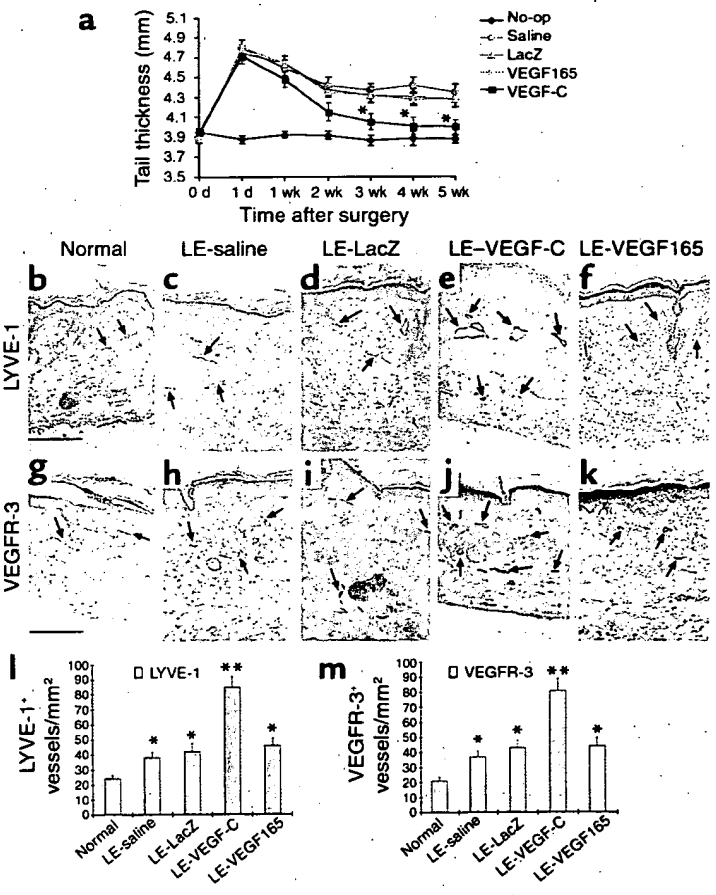

Figure 4

(**a**) Gene transfer of phVEGF-C decreases lymphedema in a mouse tail model of lymphedema. Tail thickness was significantly greater in the operated tail than in the unoperated tail during the entire 5 weeks. In the VEGF-C group, compared with the saline, LacZ, and $VEGF_{165}$ groups, the tail thickness was significantly smaller at 3–5 weeks (*P < 0.05). No-op, no operation. (**b–m**) phVEGF-C induces lymphangiogenesis in a mouse tail model of lymphedema. (**b–k**) Immunohistochemistry using markers of lymphatic endothelium, LYVE-1 (**b–f**), and VEGFR-3 (**g–k**), in normal (**b** and **g**) and operated (3 weeks after lymphedema surgery) skin sections from the saline (**c** and **h**), LacZ (**d** and **i**), VEGF-C (**e** and **j**), and $VEGF_{165}$ (**f** and **k**) groups. Lymphatic vessels are seen as brown color (black arrows). Note the abundance of hyperplastic lymphatic vessels in phVEGF-C–transfected sections (**e** and **j**). **l** and **m** show quantification of LYVE-1– and VEGFR-3–positive lymphatic vessels. Compared with normal and control (saline, LacZ, and $VEGF_{165}$) groups, the VEGF-C group showed significantly higher lymphatic vessel density. *P < 0.05 vs. normal; **P < 0.01 vs. LE-saline and LE-LacZ. Scale bar, 100 μm.

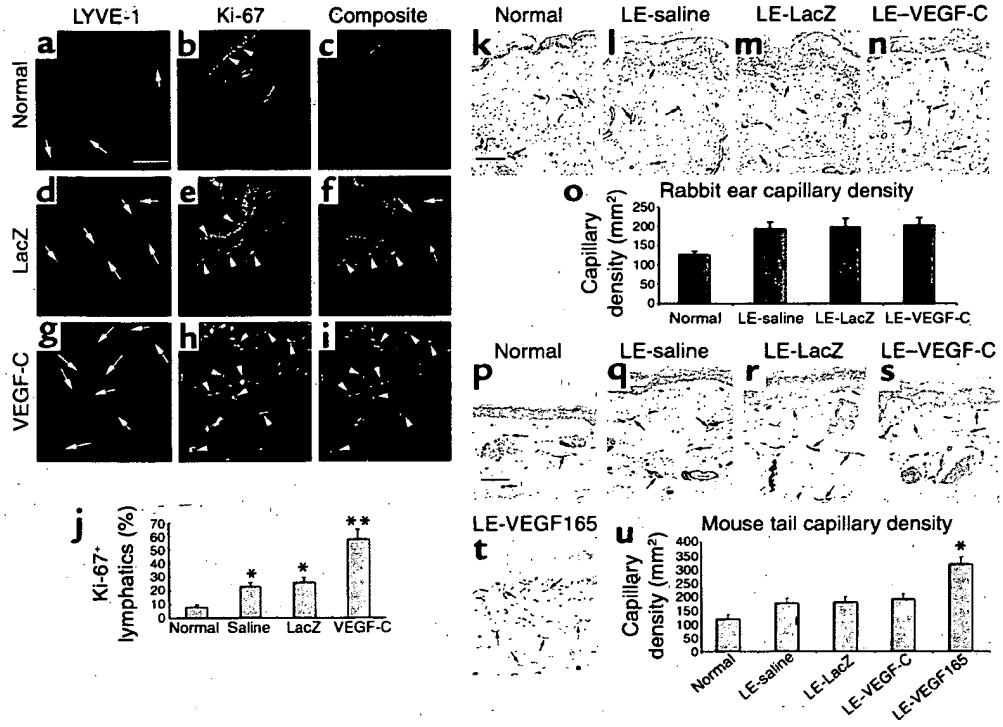

Figure 5

(**a**–**j**) phVEGF-C induces proliferation of lymphatic endothelial cells. Double immunohistochemistry using LYVE-1 and Ki-67 in active lymphangiogenesis site from skin sections. In **a**, **d**, and **g**, LYVE-1 staining of lymphatic vessels (arrows) in the dermis. In **b**, **e**, and **h**, green fluorescence (white arrowheads) depicts the nuclear staining of Ki-67. In **c**, **f**, and **i**, double fluorescence (yellow arrowheads) demonstrates Ki-67$^+$ nuclei (green) in lymphatic vessels (red). Lymphatic vessels in normal skin (**c**) are shown negative for Ki-67. In the LacZ group, some of the lymphatic vessels contain Ki-67$^+$ nuclei (**f**). White arrows in **f** show Ki-67$^-$ lymphatic vessels. In phVEGF-C–transfected skin, most of the LYVE-1–positive lymphatic vessels are positive for Ki-67 (**i**), indicating that active cell division occurs in the lymphatic vessels. (**j**) Number of Ki-67$^+$ nuclei are 2.5 times higher in the VEGF-C group. *$P < 0.01$ compared with normal; **$P < 0.01$ compared with saline and LacZ. Scale bar, 100 μm. (**k**–**u**) phVEGF-C does not increase capillary density in two animal models of lymphedema. Immunohistochemistry with CD31 (PECAM-1) in a rabbit ear (**k**–**n**) and a mouse tail (**p**–**t**) model of lymphedema on skin sections from the normal (**k** and **p**), saline (**l** and **q**), LacZ (**m** and **r**), VEGF-C (**n** and **s**), and $VEGF_{165}$ (**t**) groups. Vascular endothelial cells are stained red (black arrows). **o** and **u** show quantification of capillary density. Only the $VEGF_{165}$ group in the mouse tail model demonstrated significantly higher capillary density than the other groups. *$P < 0.01$ vs. saline, LacZ, and VEGF-C. Scale bar, 100 μm.

Results

VEGF-C gene therapy induces remission of lymphedema. Seeking to establish an appropriate animal model, initial experiments using young (6–8 months old) rabbits showed expedited regression of lymphedema such that we could not properly evaluate the effect of gene transfer. In the older rabbits (3–4 years old), a substantial degree of lymphedema developed and was sustained for more than 12 weeks.

To investigate the effect of phVEGF-C gene transfer on this lymphedema model, we measured ear thickness and volume over a 12-week period. Ear thickness was consistently smaller in the VEGF-C group than in the saline group. The difference between the groups was statistically significant beginning at 2 weeks and was maintained for the duration of the study (2 weeks: 4.5 ± 0.3 vs. 5.4 ± 0.2 mm, $P < 0.05$; 3 weeks: 3.9 ± 0.2 vs. 4.6 ± 0.3 mm, $P < 0.05$; 8 weeks: 2.8 ± 0.2 vs. 3.6 ± 0.3 mm, $P < 0.05$; 10 weeks: 2.6 ± 0.2 vs. 3.5 ± 0.3 mm, $P < 0.05$; 12 weeks: 2.4 ± 0.2 vs. 3.3 ± 0.2 mm, $P < 0.01$) (Figure 1c). Similarly, ear volume was consistently smaller in the VEGF-C group than in the saline group for the duration of the study (2 weeks: 33.1 ± 2.2 vs. 38.2 ± 1.5 ml, $P < 0.05$; 3 weeks: 29.4 ± 1.5 vs. 34.1 ± 1.0 ml, $P < 0.05$; 4 weeks: 26.5 ± 1.8 vs. 31.7 ± 1.7 ml, $P < 0.05$; 8 weeks: 18.3 ± 2.3 vs. 26.3 ± 1.9 ml, $P < 0.05$; 10 weeks: 17.1 ± 2.1 vs. 25.5 ± 1.8 ml, $P < 0.01$; 12 weeks: 14.6 ± 2.3 vs. 24.5 ± 1.3 ml, $P < 0.01$) (Figure 1d). The VEGF-C group had significantly thinner skin than the saline group at 8 weeks (2.8 ± 0.2 vs. 3.8 ± 0.2 mm, $P < 0.05$).

Lymphoscintigraphy demonstrates enhanced lymphatic drainage after VEGF-C gene transfer. In normal ears, lymphatic flow assumes a linear pattern, and the draining LNs are clearly visible at the base of the skull. (Figure 2b). Imaging performed at day 1 after surgery showed successful surgical blockade of lymphatic egress in all animals (Figure 2, c and e). Follow-up lymphoscintigraphy at 4, 8, and 12 weeks showed dynamic changes of radiotracer clearance from the operated ears that was more efficient

in the phVEGF-C–transfected ear than in the saline-injected ear. Images at 12 weeks revealed that the saline-injected ear still showed a dermal backflow pattern with faint visualization of LNs, while the phVEGF-C–transfected ear shows a linear pattern of lymphatic drainage and clear visualization of LNs (Figure 2, d and f).

Quantification of lymphatic drainage (Figure 2, g and h) over the study period revealed consistently lower retention of radioactivity in the VEGF-C group than in the saline group; this achieved statistical significance at 8 weeks (radioactivity index, 3.8 ± 0.4 vs. 5.0 ± 0.5, $P < 0.05$) and 12 weeks (radioactivity index, 2.2 ± 0.3 vs. 4.2 ± 0.4, $P < 0.05$) (Figure 2i).

Transgene expression of phVEGF-C in a rabbit ear model. To assess transgene expression of injected phVEGF-C in ear skin, we performed Western blotting of VEGF-C protein. In our experiments, two different bands were detected using two anti–VEGF-C Ab's. A 58-kDa band corresponds to the earliest processed form, while a 31-kDa band represents the major secreted form of VEGF-C polypeptides. Densitometric analysis of multiple experiments revealed that expression of the 58-kDa VEGF-C isoform in the phVEGF-C–transfected bridge was 2.6 times and 3.2 times higher than that in the saline-injected and the normal skin, respectively ($P < 0.01$) (Figure 3a). Expression of the 31-kDa VEGF-C isoform in the phVEGF-C–transfected bridge was 3.6 times and 3.9 times higher than that in the saline-injected and the normal skin, respectively ($P < 0.01$) (Figure 3b).

Gene transfer of phVEGF-C increases VEGFR-3 expression. A partial 470-bp rabbit VEGFR-3 cDNA was cloned by RT-PCR using degenerate oligonucleotide primers (GenBank accession number AF453570). The amino acid sequence displayed 92.9%, 93.6%, and 94.3% identity with human, bovine, and mouse VEGFR-3 (Figure 3c). We investigated VEGFR-3 expression using RT-PCR, revealing a nearly 1.7-fold induction of VEGFR-3 mRNA levels by VEGF-C compared with saline control ($P < 0.01$, Figure 3g).

Gene transfer of phVEGF-C increases phosphorylation of VEGFR-3 in a mouse tail model. We investigated the effect of phVEGF-C gene transfer on the tyrosyl phosphorylation of VEGFR-3 and VEGFR-2 by immunoprecipitation with anti-phosphotyrosine Ab followed by Western blot analysis with anti–VEGFR-3 and anti–VEGFR-2 Ab's, respectively. Phosphorylated VEGFR-3 (195 kDa) in the phVEGF-C–transfected samples was 1.6 times and 1.8 times higher than in the samples from the saline and LacZ groups, respectively ($P < 0.05$) (Figure 3h). Gene transfer of $phVEGF_{165}$ did not increase phosphorylated VEGFR-3 compared with the controls. Phosphorylated VEGFR-2 in the $phVEGF_{165}$–transfected sample was 2.0, 1.8, and 1.6 times higher (235-kDa band) than in the samples of the saline, LacZ, and VEGF-C groups, respectively ($P < 0.05$) (Figure 3i). Phosphorylated VEGFR-2 was slightly higher in the phVEGF-C–transfected samples than in the control groups (saline and LacZ) but was not statistically significant.

Gene transfer of phVEGF-C improves lymphedema in a mouse tail model. To determine whether the effect of phVEGF-C could be reproduced in another lymphedema model, similar experiments were performed in a mouse tail model (30) (Figure 4a). In the VEGF-C group, compared with the saline, LacZ, and $VEGF_{165}$ groups, the tail thickness was consistently smaller beginning at 3 weeks (3 weeks: 4.05 ± 0.08 vs. 4.37 ± 0.07, 4.32 ± 0.08, and 4.30 ± 0.07 mm, $P < 0.05$; 4 weeks: 4.01 ± 0.09 mm vs. 4.42 ± 0.08, 4.31 ± 0.08, and 4.28 ± 0.07 mm, $P < 0.05$; 5 weeks: 4.00 ± 0.07 mm vs. 4.35 ± 0.08, 4.28 ± 0.08, and 4.28 ± 0.08 mm, $P < 0.05$), respectively.

Gene transfer of phVEGF-C promotes lymphatic vessel growth in a mouse tail model. The VEGF-C group showed higher density of LYVE-1–positive lymphatic vessels than the other groups (VEGF-C, 85 ± 7 per mm^2; saline, 38 ± 4 per mm^2; LacZ, 42 ± 5 per mm^2; $VEGF_{165}$, 46 ± 5 per mm^2, $P < 0.01$) (Figure 4, b–f and l). Skin sections stained with VEGFR-3 Ab showed similar results (VEGF-C, 81 ± 8 per mm^2; saline, 37 ± 4 per mm^2; LacZ, 43 ± 5 per mm^2; $VEGF_{165}$, 44 ± 5 per mm^2, $P < 0.01$) (Figure 4, g–k and m). After phVEGF-C transfection, lymphatic vessels appeared hyperplastic (Figure 4, e and j). In sections at 3-week follow-up, the number of lymphatic vessels containing Ki-67$^+$ nuclei was 2.5 times higher in the VEGF-C group than in the saline or LacZ groups (VEGF-C, 58 ± 7 per mm^2; saline, 23 ± 3 per mm^2; LacZ, 26 ± 4 per mm^2, $P < 0.01$) (Figure 5, a–j).

Blood capillary density analysis. Rabbit ear and mouse tail skins were stained for an endothelial cell marker, CD31 (36). In the rabbit lymphedema model, capillary density was not significantly different among the saline (193 ± 18 per mm^2), LacZ (198 ± 22 per mm^2), or VEGF-C (201 ± 20 per mm^2) groups (Figure 5, k–o). Similar findings were observed in the capillary density of operated mouse tail groups (saline, 172 ± 18 per mm^2; LacZ, 181 ± 19 per mm^2; VEGF-C, 189 ± 20 per mm^2, *P* value not significant) (Figure 5b, F–K). However, the $VEGF_{165}$ group (302 ± 27 per mm^2) showed significantly higher capillary density than the saline, LacZ, or VEGF-C groups ($P < 0.01$).

Discussion

Chronic lymphedema is a disabling condition characterized by thickening of the skin due to fibrofatty deposition in underlying tissues as well as disfiguring swelling of affected limbs. In most cases of secondary lymphedema in humans, depletion of lymphatic vessels is the culprit in its pathogenesis (1–4). Here we show that phVEGF-C gene therapy, by promoting lymphangiogenesis, favorably modulates all the phenotypic changes associated with secondary lymphedema. We believe the present study is the first to document improvement in the clinical and pathologic features of lymphedema resulting from enhancement of lymphatic drainage by phVEGF-C gene therapy.

In two animal models, we demonstrate significant attenuation of lymphedema by phVEGF-C gene transfer.

The effect prevailed over the chronic phase as well as the acute phase of lymphedema. This improvement was also confirmed in histologic sections, which reflect chronic fibrofatty changes more accurately. Prevention or reduction of fibrotic change is one of the most important goals of therapy for lymphedema, since this secondary change can drive lymphedema into a vicious cycle by increasing interstitial solid pressure (by fibrofatty deposition) and thus collapsing already reduced or impaired lymphatic vessels (3–5, 24, 25). That the improvement in the physical indices of lymphedema was the actual result of improved lymphatic drainage was documented by quantitative lymphoscintigraphy. Although VEGF-C plasmid transgene expression is usually limited to less than 30 days (27), our results indicate that once the lymphatic connection is reestablished, the recovery of drainage function can be maintained.

VEGF-C protein expression was documented in situ after phVEGF-C gene transfer. We detected the partially processed form (58 kDa) and major secreted form (31 kDa) of VEGF-C (37, 38) after local gene delivery. VEGF-C is produced as a 61-kDa prepropeptide form which undergoes multistep proteolytic maturation. The secreted 31-kDa form predominantly activates VEGFR-3 (37). VEGFR-3 mRNA expression was very low in normal skin, slightly higher in the saline-injected experimental ears, and strongly upregulated following phVEGF-C gene transfer. We also directly measured the number of lymphatic vessels using lymphatic-specific markers. LYVE-1 or VEGFR-3 staining confirmed augmentation of lymphangiogenesis in phVEGF-C–transfected mouse tails. The hyperplastic nature of proliferating lymphatic vessels was consistent with previous reports (19, 22). Ki-67 staining documented that proliferating lymphatic endothelial cells exist in more than half the lymphatic vessels in phVEGF-C–transfected skin, suggesting a potent lymphangiogenic effect of phVEGF-C.

As VEGF-C is also known to activate VEGFR-2 and thus to induce angiogenesis in vitro and in ischemic tissues (15, 32), we evaluated capillary density from both animal models and found it was slightly higher in the VEGF-C group but not significantly different from that of the saline or LacZ control groups. The apparent absence of fully processed 21-kDa product of VEGF-C, which has potent angiogenic activity, and thus the weak activation of VEGFR-2 phosphorylation, could explain the lack of obvious angiogenesis in these animal models. These findings are compatible with previous reports that claimed no discernible angiogenesis in transgenic mice overexpressing VEGF-C in the skin (19) and in normal mouse skin that was transfected with adeno-VEGF-C (22). Physiologic function of any ligand is dependent on the temporal and spatial expression of its specific receptors. In the case of VEGF-A–induced angiogenesis, the absence of ischemia-induced regional upregulation of VEGFR-2 has been shown to result in nullification of the angiogenic effect of transient overexpression of VEGF-A (39, 40). To further address the concern that increased angiogenesis might improve lymphedema, we investigated the effect of VEGF-A ($phVEGF_{165}$) plasmid gene transfer in the mouse tail model and found that augmenting angiogenesis but not lymphangiogenesis did not improve lymphedema.

The potential clinical relevance and limitations of our study derive from certain features of the design and the findings. First, the models are pathophysiologically similar to the secondary forms of human lymphedema. The models we used do not represent the entire spectrum of lymphedema found in humans, especially the primary form of lymphedema, which is an inherited developmental disorder of the lymphatic system. However, pathophysiologically our models are approximations of secondary lymphedema, which comprises most cases of lymphedema and results primarily from surgical removal of lymphatic vessels and lymph nodes in industrialized countries (1–4). Additionally, in our models, we used gene therapy in an acute/subacute stage of lymphedema. Therefore, whether this gene therapy can be effective in chronic cases is uncertain. As there are concerns about the potential enhancement of tumor growth and metastasis by VEGF-C in tumor models (41), we need to consider the potential advantages and dangers of using local VEGF-C therapy in patients with lymphedema caused by cancer treatment. This issue can be resolved after performing experiments adopting tumor implantation and treatment such as a combination of surgery, chemotherapy or radiation, and local VEGF-C gene transfer. Third, the approach of gene therapy using naked plasmid DNA (phVEGF-C) has been used in early clinical trials and has an accumulating record of safety (42). Finally, to the best of our knowledge, this study represents the first experimental proof of a beneficial effect of VEGF-C gene therapy on lymphedema per se. Our findings clearly indicate a favorable effect of phVEGF-C–induced lymphangiogenesis on lymphedema and thus represent a novel therapeutic paradigm for the treatment of this otherwise difficult-to-manage condition.

Acknowledgments

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RESEARCH ARTICLE

Adenovirus-mediated $VEGF_{165}$ gene transfer enhances wound healing by promoting angiogenesis in CD1 diabetic mice

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It has been previously shown that vascular endothelial growth factor (VEGF) plays a central role in promoting angiogenesis during wound repair and that healing-impaired diabetic mice show decreased VEGF expression levels. In order to investigate the potential benefits of gene therapy with growth factors on wound repair, a replication-deficient recombinant adenovirus vector carrying the human $VEGF_{165}$ gene (AdCMV.$VEGF_{165}$) was topically applied on excisional wounds of streptozotocin-induced diabetic mice. Treatment with AdCMV.$VEGF_{165}$ significantly accelerated wound closure when compared with AdCMV.LacZ-treated, as well as saline-treated control mice, by promoting angiogenesis at the site of injury. Our findings suggest that AdCMV.$VEGF_{165}$ may be regarded as a therapeutic tool for the treatment of diabetic ulcers.

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Keywords: *VEGF; gene therapy; wound healing; diabetes*

Introduction

Wound healing is a complex process which encompasses three phases: inflammation, granulation tissue formation and tissue remodeling.[1] These events are triggered by cytokines and growth factors which are released at the site of injury. Angiogenesis, the sprouting of new blood vessels from a pre-existing network,[2] is necessary to allow migration of leukocytes and growth factors and oxygen supply during granulation tissue formation. Once granulation tissue completely replaces the initial fibrin clot, tissue remodeling begins and the new vessels start to regress.

Vascular endothelial growth factor is one of the most important endothelial growth factors and it is a dimeric glycoprotein characterized by the six isoforms $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$, $VEGF_{206}$, derived from alternative splicing.[3] It has mitogenic activity on endothelial cells *in vitro*, shows angiogenic properties *in vivo*[4,5] and enhances vascular permeability of blood vessels.

VEGF plays an important role in the induction of angiogenesis during wound healing and its expression is up-regulated by hypoxia both *in vivo* and *in vitro*.[6] In adult skin, VEGF expression is almost undetectable under physiologic conditions, while its up-regulation occurs in hyperplastic epidermis of healing wounds[7] and psoriatic skin.[8] VEGF is a potent mitogen for dermal microvascular endothelial cells,[9] it is expressed in keratinocytes of healing wounds[10,11] and acts in a paracrine manner on dermal microvessels, leading to increased skin vascularity.[12,13] Recently, the *in vivo* activity of hVEGF promoter has been shown in wounded transgenic mouse skin.[14]

Elevated VEGF mRNA levels are found in normal mice during granulation tissue formation, while levels are significantly reduced in db/db diabetic mice.[11] A similar reduction of VEGF gene expression has been reported in non-obese diabetic mice in response to hindlimb ischemia. Since genetically and streptozotocin-induced diabetic mice show impaired wound healing when compared with healthy animals,[15–17] it is possible that this may reflect, at least in part, an inadequate VEGF expression.

Therefore, to address the importance of VEGF *in vivo* in skin angiogenesis during wound repair in diabetes and to identify a potential therapeutic tool, we investigated the effect of gene therapy with an adenoviral vector carrying the $VEGF_{165}$ gene on excisional wound healing in streptozotocin-induced diabetic mice.

Our results show that adenovirus-mediated $VEGF_{165}$ gene transfer induces accelerated wound healing by promoting angiogenesis at the site of injury and suggest that topical $VEGF_{165}$ gene transfer should be evaluated for clinical application for the treatment of chronic nonhealing wounds in diabetic patients.

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Results

AdCMV.VEGF$_{165}$ transduction in skin wounds

Replication-deficient recombinant adenovirus vectors can transfer genes to several cell types both *in vitro* and *in vivo*.[18–20] Furthermore, they can transfer genes directly to skin cells *in vivo*.[21] We first assessed whether an adenovirus vector carrying the VEGF$_{165}$ cDNA could efficiently transduce mouse skin after its topical application. Western blot analysis was performed on cell lysates from wounds treated either with the control vector AdCMV.LacZ or AdCMV.VEGF$_{165}$; exposure to the adenovirus vector occurred either 48 or 72 h before the death of the animals. As shown in Figure 1, VEGF$_{165}$ was abundantly expressed in transduced skin while trace amounts, corresponding to endogenous VEGF, ie murine VEGF$_{164}$, were present in AdCMV.LacZ-treated control samples.

VEGF$_{165}$ enhances wound healing in diabetic mice

Analysis of wound closure of AdCMV.VEGF$_{165}$-, AdCMV.LACZ-, saline-treated and nondiabetic CD1 mice was performed through digital processing of pictures taken at the time of wounding (time 0) and at day 2, 3, 5, 7, 9, 11 and 13 days after skin damage, ie until wound closure was complete in all groups. A representative example of wound closure of treated and control animals is shown in Figure 2a. Average results (Figure 2b) show that treatment of mice with AdCMV.VEGF$_{165}$ accelerated wound healing when compared with treatment with control vector or to the uninfected control (saline). Interestingly, diabetic AdCMV.VEGF$_{165}$-treated mice healed similarly to nondiabetic CD1 mice (Figure 2b). A statistically significant difference between AdCMV.VEGF$_{165}$-treated mice and both AdCMV.LACZ- and saline-treated mice was already apparent at day 3 after treatment and persisted at all time-points evaluated from day 3 to day 9. It is noteworthy that at day 3 wound healing was accelerated in AdCMV.VEGF$_{165}$-treated mice also *versus* nondiabetic CD1 mice. However, at later time-points this difference disappeared.

Effect of AdCMV.VEGF$_{165}$ on granulation tissue formation

In order to assess the effect of AdCMV.VEGF$_{165}$ on the repair process, wounds were examined histologically and granulation tissue area was measured. Compared with AdCMC.LacZ- and saline-treated mice, animals treated with 10^8 p.f.u. of Ad.CMV.VEGF$_{165}$ showed higher

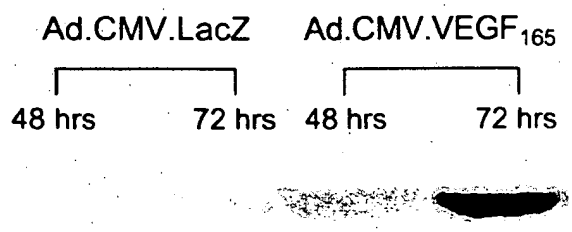

Figure 1 *Expression of VEGF$_{165}$ protein by wounded skin following adenoviral vector transduction. Mouse wounded skin was examined for VEGF$_{165}$ protein expression after topical application of AdCMV.LacZ (lanes 1, 2) and AdCMV.VEGF$_{165}$ (lanes 3, 4) at time 0. VEGF expression was analyzed at 48 h (lanes 1, 3) or 72 h (lanes 2, 4) following infection.*

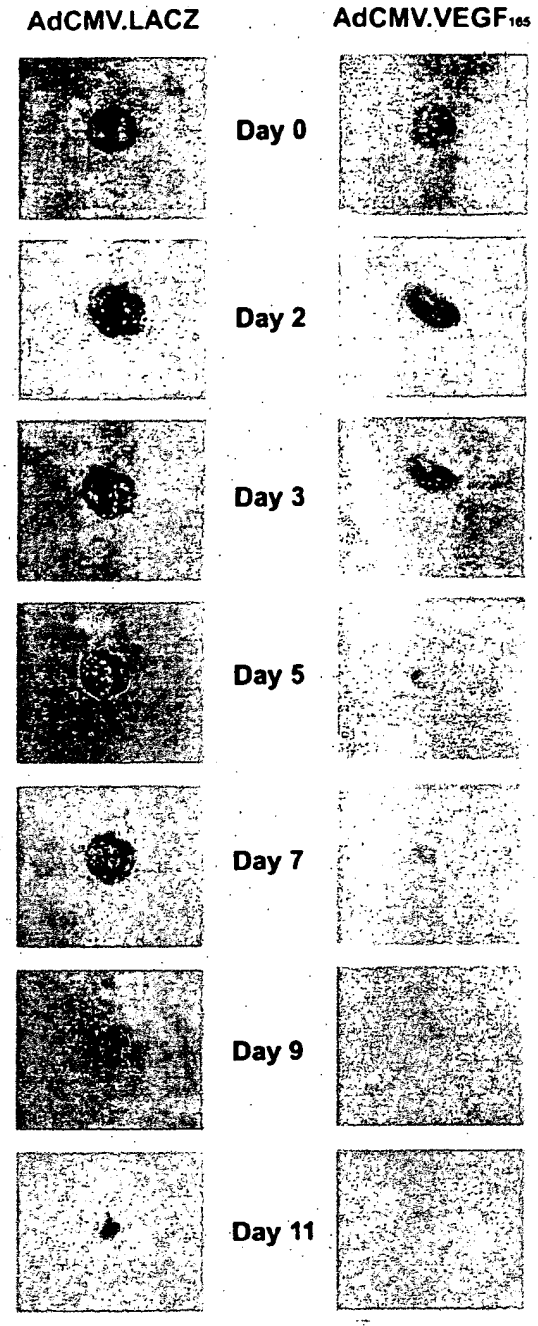

Figure 2a *Excisional wound healing of CD1 streptozotocin-induced diabetic mice treated with adenoviral vectors. (a) Representative examples show wound healing of mice treated either with AdCMV.LacZ or AdCMV.VEGF$_{165}$. Wounds were photographed at the time indicated from days 0 to 11. Day 0 pictures were taken immediately after wounding.*

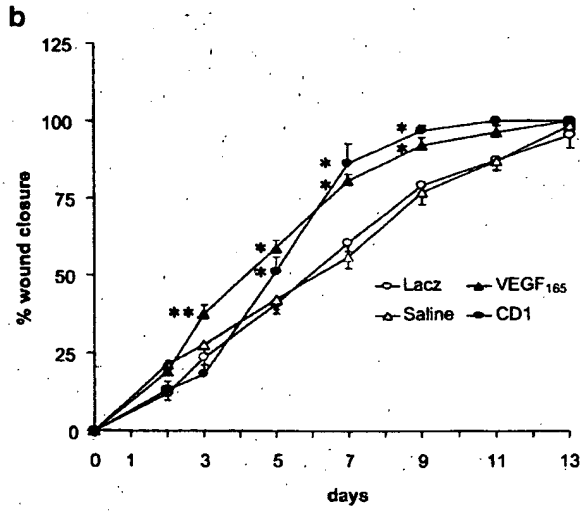

*Figure 2b Average results show the rate of wound healing in diabetic mice treated either with AdCMV.LacZ, AdCMV.VEGF$_{165}$ or saline and in nondiabetic CD1 mice. VEGF$_{165}$ gene transfer enhanced wound healing in comparison to control diabetic mice. Each time-point represents the mean of the results obtained in seven to 29 mice; *indicates $P < 0.05$ versus LacZ and saline, **indicates $P < 0.05$ versus CD1, LacZ and saline. Average wound area at time 0 was about 11 mm^2 and comparable among all groups.*

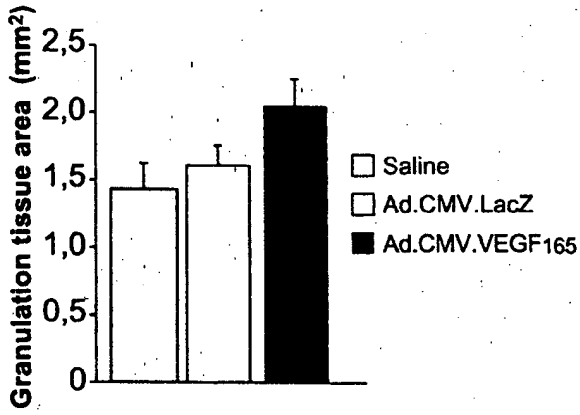

Figure 3 Effect of AdCMV.VEGF$_{165}$ topical application on granulation tissue formation. Central sections of wounds were examined at day 5 from skin damage to assess the amount of granulation tissue area following AdCMV.LacZ or AdCMV.VEGF$_{165}$ or saline treatment.

amounts of granulation tissue at day 5 after wounding (Figure 3).

VEGF$_{165}$ promotes angiogenesis in wounds

In order to investigate whether the ability of AdCMV.VEGF$_{165}$ to accelerate wound closure was associated with an angiogenic effect at the site of injury, PECAM-1 staining was performed on central sections 7 days after wounding. This staining allowed estimation of the density of PECAM-1-positive vessels in AdCMV.VEGF$_{165}$ treated mice (Figure 4c) *versus* control mice (Figure 4a and b). A two- and a 3.7-fold increase in blood vessel density was observed in AdCMV.VEGF$_{165}$- *versus* AdCMC.LacZ- and saline-treated mice, respectively (Figure 4d). In order to further analyze the angiogenic response to VEGF treatment, the arteriolar density was measured by means of α-smooth muscle actin immunostaining and by morphometric analysis. Such analysis revealed that 7 days after skin wounding and topical application of adenoviral vectors or saline, a significant increase occurred in the length density of arterioles with a luminal diameter of 4 to 41 μm in AdCMV.VEGF$_{165}$- *versus* AdCMC.LacZ- and saline-treated mice (two-fold and 3.1-fold, respectively) (Figure 5). The angiogenic effect of VEGF may be underestimated by the increase in vascular permeability induced by the growth factor.

Discussion

The results of the present study show that adenovirus-mediated VEGF$_{165}$ gene transfer to excisional wounds in diabetic mice enhances healing and is associated with a significant angiogenic response.

Skin ulcers, mostly affecting the foot, are a common complication of diabetes mellitus[22] and they can be due to different causes which frequently coexist. Atherosclerosis of large and medium-sized arteries and microangiopathy affecting small vessels cause ischemic ulcers.[23] Nevertheless, other patients develop neurotrophic ulcerations in the absence of ischemia. Both ischemic and neurotrophic ulcerations can be complicated by localized infection and heal with considerable difficulty.

Altered wound repair is present in animal models of diabetes such as non-obese diabetic mice (NOD), db/db mice[15–26] and mice in which the diabetes was induced by treatment with streptozotocin. NOD mice develop a form of diabetes similar to human type-I insulin-dependent diabetes, also known as type 1 diabetes, that is an organ-specific autoimmune disease resulting from the destruction of insulin-producing pancreatic β cells.[24] In NOD mice impairment of angiogenesis and a significant reduction of VEGF expression compared with VEGF levels in normal C57 mice have been documented. Specifically, the impairment of angiogenesis was characterized by decreased capillary density and significant blood flow reduction in ischemic hindlimbs.[25]

Diabetes in the C57 BL7KSJ db mouse (db/db mouse) is similar to maturity-onset diabetes (non-insulin-dependent diabetes mellitus, NIDDM) in humans and is characterized by obesity, infertility, hyperphagia and marked hyperglycemia.[26] Db/db mice show a significant reduction of VEGF mRNA expression during the healing process.[11]

Treatment of mice with streptozotocin induces an insulin-deficient diabetes that is similar to type I diabetes and several strains of mice including CD1 have been rendered diabetic by this method.[27,43]

VEGF may have both direct and indirect effects on wound healing by increasing endothelial cell proliferation and vascular permeability, thus allowing the delivery of plasma proteins and influx of migrating cells to the site of injury.[28]

Further, *in vitro* studies have shown that a high glucose concentration leads to decreased endothelial cell prolifer-

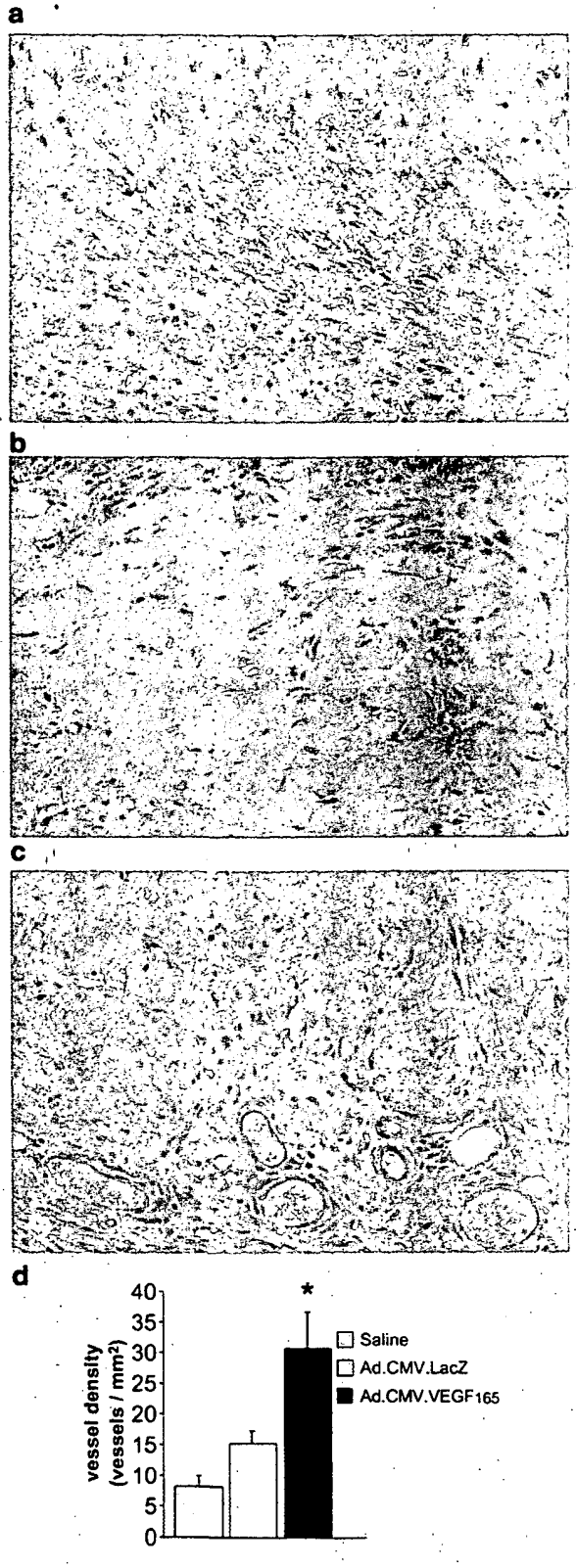

Figure 4 Determination of skin vessel density by immunohistochemical analysis. Total vessel density was determined 7 days after wounding by anti-PECAM-1 staining (×200 magnification). Representative staining of (a) saline-treated, (b) AdCMV.LacZ-treated and (c) AdCMV.VEGF$_{165}$-treated wounds. (d) Total vessel density was significantly increased in VEGF$_{165}$-treated as compared with saline- and AdCMV.LacZ-treated wounds ($P < 0.05$).

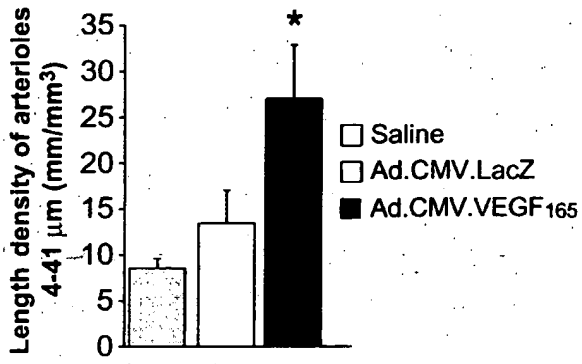

Figure 5 Evaluation of arteriolar density in treated mice. Arterioles length density in wounded skin measured by α-smooth muscle actin staining showed a significant increase of arterioles length density in mice treated with AdCMV.VEGF$_{165}$ as compared with saline- and Ad.CMV.LacZ-treated mice ($P < 0.05$).

ation and differentiation, as well as cell cycle and morphological alterations of endothelial cells.[29,30]

The expression of VEGF and VEGF receptors is upregulated, during the normal wound healing process, in migrating keratinocytes,[31,32] in sprouting blood vessels at the wound edge and in the granulation tissue. VEGF production is stimulated during this process, besides by hypoxia, by key molecules such as epidermal growth factor (EGF) family members, keratinocyte growth factor (KGF) and transforming growth factor-β1 (TGF-β1)[11] accumulating at the wound site.

In order to enhance wound repair, supplementation of recombinant angiogenic growth factors, such as nerve growth factor (NGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) 2 and granulocyte–macrophage stimulating factor (GM-CSF) has been used both in animal models[15,33] and in clinical trials of patients with chronic ulcers.[34,35] Treatment with recombinant growth factors has often been successful in increasing re-epithelialization, granulation tissue formation and extracellular matrix deposition, thus enhancing wound healing. On the other hand, short half-life of these growth factors, mostly due to the activity of tissue proteases, and their elevated costs, have limited their use in clinical trials.

Therapeutic angiogenesis with angiogenic factors inserted into plasmids or adenoviral vectors has been widely utilized in both animal models and in clinical trials to ameliorate peripheral,[25] cardiac ischemia[36,37] and wound repair.[38–40] VEGF gene transfer has been shown to enhance angiogenesis in animal models of hindlimb and cardiac ischemia and under these experimental conditions, it improves blood flow to the ischemic tissues.[25,36,41]

In the present study VEGF$_{165}$ gene transfer, in the absence of experimentally induced ischemia, significantly

improved wound healing in diabetic animals. Topical application into excisional wounds resulted in efficient $VEGF_{165}$ expression in mouse skin, where it induced an accelerated rate of closure by promoting angiogenesis at the site of injury. Treatment with AdCMV.$VEGF_{165}$ induced a rate of wound closure in diabetic mice similar to the one observed in CD1 nondiabetic mice thus indicating the efficacy of such treatment. On the other hand, a slower and comparable closure rate was shown in both AdCMV.LacZ- and saline-treated diabetic mice, suggesting that the accelerated healing of VEGF-treated animals was not secondary to the inflammatory response elicited by treatment with the adenoviral vectors.

In this study we show that $VEGF_{165}$ treatment induces healing by promoting neoangiogenesis at the site of injury. VEGF overexpression may have exerted a direct mitogenic effect on endothelial cells and also increased vascular permeability and macrophage infiltration.[42]

In conclusion, adenovirus-mediated gene transfer of excisional wounds with $VEGF_{165}$ may be regarded as a therapeutic tool for the treatment of diabetic ulcers. Additional studies will be required to determine the clinical applicability of $VEGF_{165}$ gene transfer to improve wound healing in diabetic patients.

Materials and methods

Adenoviral vectors

The replication-deficient adenovirus vector containing the cDNA for human $VEGF_{165}$ (AdCMV.$VEGF_{165}$) has been previously described.[43] AdCMV.LacZ carries the cDNA for the *E. coli* LacZ gene and was used as a control vector.

Animal wound model

CD1 male mice were obtained from Charles River (Calco, Italy). These mice were rendered diabetic by intraperitoneal injection of streptozotocin (Sigma-Aldrich, St Louis, MO, USA) at 1.2 mg/30 g weight/day for 5 consecutive days.[44] After 7 days, glycemia was measured and animals with glycemia of 200 to 400 mg/dl were selected for further studies. Mice were anesthetized with i.m. injection of 2.5% avertin (100% Avertin: 10 g of 2,2,2-tribromoethyl alcohol (Sigma) and 10 ml of tert-amyl alcohol, (Sigma)). The dorsum was clipped free of hair and a full-thickness wound of 3.5 mm diameter was created using a biopsy punch.

Adenovirus-mediated gene transfer (AdCMV.$VEGF_{165}$ or AdCMV.LacZ) was performed by placing 10^8 p.f.u. in 10 µl of PBS directly on the wound. Another group of diabetic mice was treated by placing 10 µl of PBS on the wound.

Adenovirus transduction efficiency in wounded mouse skin

Animals were killed 48 h and 72 h after treatment, and wounded areas were surgically removed. Skin samples were trimmed with small scissors on ice and 3 ml of RIPA buffer (1 × PBS, 1% NP40, 150 mM NaCl, 0.1% SDS) containing proteinase inhibitors (PMSF, aprotinin and sodium orthovanadate) were added to each gram of tissue sample. Tissues were then homogenized on ice using a blender (PBI International, Milano, Italy), incubated for 30 min on ice and centrifuged at 14 000 r.p.m. at 4°C. Supernatants were recovered and centrifuged again at 14 000 r.p.m. (Eppendorf Netheler, Hinz Gmbh, Germany). Protein concentration was measured using a Bradford assay.

For Western blot analysis, 60 µg of each sample were run on a 12% polyacrylamide gel. The gel was then transferred on to nitrocellulose membrane (S&S, Amersham Pharmacia Biotech, UK) following standard procedures, and $VEGF_{165}$ expression was detected using a specific polyclonal rabbit antibody (1:100 dilution) (R&D, Minneapolis, MN, USA) and a secondary HRP-anti-rabbit antibody (1:10 000 dilution) for ECL detection (Amersham) following standard procedures.

Determination of wound closure rate

To compare the rate of wound closure between AdCMV.$VEGF_{165}$-treated, AdCMV.LacZ-treated and saline-treated diabetic mice, as well as CD1 non-diabetic mice, animals were photographed at day 0, 2, 3, 5, 7, 9, 11 and 13 after treatment. Pictures were digitally processed and areas of wounds were calculated using the KS300 system (Zeiss, Jena, Germany). For each sample the rate of the healing process was measured as a ratio of the area at each time-point divided by the area at time 0 (ie immediately after the wound). Seven to 29 animals/group were analyzed as a total of six independent experiments.

Histology

Wounds and surrounding skin areas were surgically removed, fixed in 10% buffered formalin for 48 h at room temperature and embedded in paraffin. Sections (4 µm thickness) were mounted on glass slides treated with poly-L-lysine (Sigma) and subjected to hematoxylin-eosin (H-E) staining following standard techniques. Serial sections were prepared for each specimen and central sections of the wounds were identified by measuring the largest epithelial gap, ie the maximal distance between the epithelial edges. Sections were then subjected to immunohistochemical analysis and quantification of granulation tissue by two independent investigators.

H-E staining of the granulation tissue areas was performed on diabetic mice treated with AdCMV.$VEGF_{165}$, AdCMV.LacZ or saline and killed 5 days after wounding. The areas were calculated with the Zeiss image analyzer KS300, at ×50 magnification and were expressed in mm^2.

Immunohistochemistry and morphometric analysis

Immunohistochemical staining of the central region of wounds of treated mice, $n = 5$ in each group, killed on day 7 after wounding, was performed. Briefly, sections (4 µm thickness) were processed with xylene, passed sequentially through graded alcohol, PBS-washed and then incubated with anti-PECAM-1 antibody (1:200 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature (RT). A biotinylated rabbit anti-goat immunoglobulin was used as secondary antibody (1:50 dilution) (Vector Laboratories, Peterborough, UK) for 1 h at RT, followed by treatment with avidin biotin complex (1:100 dilution) (Vectors Laboratories) for 1 h at RT. The sections were rinsed for 15 min in PBS and color development was performed with 3,3'-diaminobenzidine for 5 min. Sections were finally counterstained with hematoxylin to identify nuclei. A rabbit serum (1:10 dilution) was used as a negative control.

Vessel density was measured as a number of PECAM-1-positive vessels (×400 magnification) divided by the total area (mm^2) of the central section by two independent investigators. The areas were calculated with the Zeiss image analyzer KS300.

Morphometric analysis was performed at day 7 after wounding. Five mice treated with either AdCMV.LacZ, AdCMV.VEGF$_{165}$ or saline were killed, skin samples were taken and fixed in formalin. The central sections were stained with anti-α-smooth muscle actin antibody in order to identify intermediate-sized arteries and arterioles and to distinguish them from capillaries and veins. Briefly, sections were deparaffinized, rinsed with PBS, incubated at 37°C for 60 min with a monoclonal anti-α-smooth muscle actin (clone 1A4, Sigma) (1:30 dilution), and subsequently incubated at 37°C for 1 h with anti-mouse IgG tetramethylrhodamine B isothiocyanate (TRITC)-labeled antibody diluted 1:60 in PBS. Finally, sections were rinsed in PBS and mounted in Vectashield (Vector Laboratories). For morphometric analysis, the skin total area was examined (×400 magnification) and the length of major and minor luminal diameters and wall thickness along the minor axis of each artery and arteriole were analyzed.[45] For n profiles counted in an area A, the length density (Ld) is equal to the sum of the ratio of the major or long axis to the minor or wide axis of each profile. Thus, Ld is equal to the length per unit volume in the same dimensional area: $Ld = 1/A\ \Sigma R = (R_1 + R_2 + R_3 + .. R_n)/A$. Arteriole length density was expressed per unit of volume (mm/mm^3) of skin.[45,46]

Statistical analysis

All data are expressed as means ± s.e.m. Comparisons among different groups for continuous variables were carried out by analysis of Variance (ANOVA). Post-hoc pairwise comparisons were performed, when appropriate (ANOVA $P < 0.05$), using the Student–Newman-Keuls method. A P value of <0.05 was considered statistically significant.

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Enhancement of expression of vascular endothelial growth factor after adeno-associated virus gene transfer is associated with improvement of brain ischemia injury in the gerbil

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Abstract

Angiogenesis induced by growth factors may represent a rational therapy for patients with stroke. Vascular endothelial growth factor (VEGF) plays a pivotal role in angiogenesis and VEGF expression is enhanced in the post-ischemic brain. VEGF induced by brain hypoxia can lead to the growth of new vessels and may represent a natural protective mechanism improving survival after stroke.

In the light of these findings we investigated changes of VEGF expression in different brain regions after intracerebroventricular injection of adeno-associated virus transfering gene for VEGF (rAAV-VEGF) in the gerbil, and after transient brain ischemic injury, we studied the effects of rAAV-VEGF injection on survival, brain edema, delayed neuronal death in the CA1 area and learning ability.

Treatment with rAAV-VEGF 6 days or 12 days before ischemia significantly improves survival, brain edema and CA1 delayed neuronal death and post-ischemic learning evaluated by passive avoidance test. Animals treated with rAAV-VEGF showed in the thalamus and the cortex, a significant positive immunostaining for VEGF similar to those subjected to brain ischemia and not treated with rAAV-VEGF.

These data represent a further contribution to a possible employment of gene therapy by using rAAV-VEGF in brain ischemia and indicate that thalamus and cortex may be targets for neuroprotective effects of VEGF.

Keywords: Vascular endothelial growth factor; Gene therapy; Brain ischemia; Passive avoidance; Gerbil; Brain injury

1. Introduction

Vascular endothelial growth factor (VEGF) is a 45-kDa homodimeric glycoprotein. It is a potent mitogen for micro- and macrovascular endothelial cells derived from arteries, veins and lymphatics [1]. VEGF promotes a strong angiogenic response [2] and it also acts as a permeability factor [3,4]. This factor has high affinity for two receptors, *fms-like* tyrosine kinase (flt-1) and the kinase domain region (KDR) [5,6]. VEGF induces dissolution of the extracellular matrix, causes chemotaxis and proliferation of capillary endothelial cells and also promotes tube proliferation of endothelial cells. There are existing four different isoforms, $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$, generated by alternative splicing of mRNA. It is produced by keratinocytes that represent with macrophages the most important source of VEGF [7].

Hypoxia is a strong inducer of VEGF mRNA expression in many cells in vitro [8,9]. In addition, in vitro experiments have revealed that systemic hypoxia is capable of inducing the expression of both VEGF and VEGF receptor in various organs including the brain [10].

It has been shown that VEGF administration improves myocardial perfusion and enhances angiogenesis either in humans affected by ischemic coronary disease [11] or in animal models of myocardial and limb ischemia [12].

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In vitro and in vivo studies indicate that VEGF is also involved in brain ischemia physiopathology. Addition of VEGF protects primary cultured cortical neurons of rat from hypoxia and glucose deprivation [13]. VEGF expression in the cortex of the rat is enhanced after transient global brain ischemia [14]. Moreover, it has been also demonstrated that administration of VEGF can markedly increase angiogenesis in the ischemic brain and reduces neurological deficit during stroke recovery [15].

Recently, numerous studies have reported that the adenovirus expression vector is useful for application to the gene therapy. With the aim to evaluate the protective effect of VEGF gene therapy in transient brain ischemia we used adeno-associated virus (AAV) as a vector for VEGF delivery.

2. Methods

2.1. rAAV vector preparation and characterization

Two recombinant AAV vectors were obtained in this study, expressing the LacZ reporter gene (control gene) and the cDNA for the 165 amino-acid isoform of VEGF (VEGF165) under the control of the strong and constitutive cytomegalovirus (CMV) immediate early promoter. Both constructs were based on plasmid pFU-5, kindly provided by N. Muzyczka (University of Florida, Gainesville, FL). The rAAV-LacZ was obtained by substituting the GFP open reading frame with the LacZ gene from plasmid pCH110 (Pharmacia, Uppsala, Sweden). The cDNA for VEGF165 was obtained by RT-PCR amplification of HL-60 total RNA with appropriate primer pairs and again cloned to replace GFP in the pFU-5 vector digested with Bam HI and Eco RI. Infectious vector stocks were generated in 293 cells, cultured in 150-mm diameter Petri dishes, by co-transfecting each plate with 15 ng of each vector plasmid together with 45 ng of the packaging/helper plasmid, pDG (kindly provided by J.A. Kleinschmidt), expressing AAV and adenovirus helper functions [9]. Twelve hours after transfection, the medium was replaced by fresh medium and 3 days later the medium was collected and the cells harvested by scraping. After three freeze–thaw cycles in dry ice/ethanol bath and 37 °C water bath, cell lysates were fractionated using ammonium sulfate precipitation. The rAAV particles were then purified by CsCl gradient centrifugation in a SW41Ti rotor at 288,000 × g for 36 h. Twelve to sixteen fractions of ten drops each were collected by inserting a G-21 needle below the rAAV band and their refractive index was determined. The six fractions with index closest to 1.3715 (corresponding to a density of 1.40 g/cm^3) were dialyzed against phosphate buffered saline (PBS) at 4 °C overnight and stored at −80 °C. rAAV titers were determined by measuring the copy number of viral genomes in pooled, dialyzed gradient fractions. This was achieved by a competitive PCR procedure [16] using primers and competitors mapping in the CMV promoter region common to all vector. The purified viral preparations used for this work had particle titers of $\sim 1 \times 10^{12}$ viral genomes per milliliter.

2.2. Animals

Adult male Mongolian gerbils weighing 60–70 g were used. Before and after ischemia/reperfusion they were housed four to a cage at a constant room temperature of 21–22 °C under a light cycle of 12 h/12 h (7.00 a.m./7.00 p.m.). Animals were allowed free access to food and drinking water. Adaptation and experiments were carried out in accordance with the internationally accepted principles and the national laws concerning the care and the use of laboratory animals and in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

2.3. Injection of vector into gerbil brain

Gerbils were anesthetized with chloral hydrate (2.5%), placed in a stereotaxic apparatus and a guide cannula was implanted into the left lateral cerebral ventricle according to the atlas of Thiessen and Yahr [17]. Through the guide cannula, 6 or 12 days before brain ischemia or sham-operation, animals were treated in the left lateral cerebral ventricle with rAAV-LacZ (control) or with rAAV-VEGF (0.5–25 U) injected in 10 ml of artificial cerebrospinal fluid.

2.4. Surgery

Experimental model of transient brain ischemia in the gerbil was used. This experimental model does not reproduce totally human pathophysiology, but it is widely used and can purchase useful data on neuroprotective agents. The ischemia/reperfusion injury was induced by a single

Table 1
Effects of pre-treatment (6 or 12 days before ischemia) with rAAV-VEGF or rAAV-LacZ on survival of gerbils subjected to transient bilateral carotid occlusion

Treatment	Number of animals	Survivors	Percentage of survivors
rAAV-LacZ 6 days before ischemia	24	18	75
rAAV-LacZ 12 days before ischemia	24	18	75
rAAV-VEGF 6 days before ischemia	24	21[a]	87.5
rAAV-VEGF 12 days before ischemia	24	22[a]	91.7

[a] vs. rAAV-LacZ, 6 and 12 days before ischemia.

3-min bilateral occlusion of the common carotid arteries initially (about 4–5 min) under halothane (5%) anesthesia followed by nitrous oxide/O_2 (70% N_2O/30% O_2); surgery was performed under nitrous oxide/O_2 and halothane was decreased to 2%. Adequacy of the anesthesia was monitored by foot pinch. Total period of anesthesia was about 18 min. Sham-operated animals, not exposed to ischemic insult, served as controls. Body temperature was maintained at 36–37 °C during the ischemic and the immediate post-ischemic period with a homeothermic blanket. Surgery was always done between 10.00 and 12.00 a.m. Survival of animals was evaluated within the first 24 h after the surgery (Table 1).

3. Evaluation of brain edema

To evaluate the extent of brain edema following cerebral ischemia, tissue sections from one group of animals were assayed for water content at 48 h after injury, using wet weight/dry weight ratios. Freshly dissected tissue samples of hippocampus were weighed on aluminium foil, dried for 24 h at 105 °C, and reweighed as previously [18].

The percentage of water was calculated as follows:

$$\%\,\text{water} = \frac{\text{wet weight} - \text{dry weight}}{\text{wet weight}} \times 100$$

3.1. Immunohistochemistry

Immunohistochemistry was performed 6 or 12 days after brain ischemia or after rAAV-VEGF injection in animals as follows: one group of not ischemic and treated with rAAV-LacZ gerbils (control) 6 or 12 days after brain ischemia; a second group of animals subjected to transient brain ischemia and sacrificed 6 or 12 days after ischemia; a third group of not ischemic animals treated with rAAV-VEGF and sacrificed 6 or 12 days after rAAV-VEGF injection. When animals were sacrificed animals were perfused through the ascending aorta with saline followed by a solution of paraformaldehyde 4% in 0.1 M phosphate buffer (PB).

Forty-micrometer thick brain coronal sections were cut by a freezing microtome and stored in PB at 4 °C. A set of sections, regularly spaced through the brain, was mounted and stained with thionin 0.25% for histological identification of nervous structures by analogies with the brain of the rat, according to the atlas of Thiessen and Yahr [17]. The remaining sections were used for immunohistochemical experiments. Primary antisera for VEGF was used (Santa Cruz Biotechnology, Inc.). Free-floating sections through the brain were rinsed in tris-buffered saline of pH 7.6 (TBS), and incubated overnight with the primary antibodies diluted in TBS containing 2% normal goat serum (NGS) as follows: VEFG 1:500. Sections were then rinsed and incubated for 60 min with biotinylated Goat–Anti-Rabbit serum (diluted 1:200 in TBS containing 1% NGS; 60 min with avidin-biotinylated peroxidase complex (diluted 1:100 in TBS) and 4–8 min with a solution of 3,3-diaminobenzidine tetrahydrocloride 0.5 M Tris buffer containing hydrogen peroxide as chromogen to visualize peroxidase activity. No immunostaining was observed in control tissue where: (a) the primary antiserum was omitted; (b) the primary antiserum was substituted by normal rat serum, (c) the biotinylated antiserum, avidinbiotinylated peroxidase complex or any component of the chromogen solution was omitted. Sections were finally mounted, air dried and cover slipped for microscopic observation.

Quantitative estimates of immunostaining were calculated by computer-assisted densitometry. For this purpose, the optical density of images was analyzed in a range of 256 gray level values: value "zero" was assigned to a bright area of the slide void tissue; value "one" was assigned to black. Calibration of intermediate values was obtained with a series of neutral density filters of known values. Microscope illumination and parameters of the video-amplifier were carefully adjusted to avoid saturation of the images. These conditions were kept constant.

3.2. Delayed neuronal death

After performing the passive avoidance tests, animals were killed by decapitation under anesthesia with chloral hydrate. The brains were harvested and preserved in 10% buffered neutral formalin for 10 days. Formalin-fixed brain blocks containing dorsal hippocampus were embedded in paraffin. Thick sections (5 μ) were sliced at the level of the dorsal hippocampus and stained with hematoxylin and eosin for light microscopic examination. The grading system of Pulsinelli et al. [19] was used: grade 0 = 0% of the neurons damaged (normal brain), grade 1 = 1–10% of the neurons damaged, grade 2 = 11–50% of the neurons damaged, grade 3 = more than 50% of the neurons damaged, and grade 4 = infarction (necrosis of both neurons and glia). The slices were evaluated independently by two examiners.

3.3. Passive avoidance

The gerbils were trained in a step-through type passive avoidance apparatus 7 days after brain ischemia. The passive avoidance test-setup is divided into two sections. One compartment is white and illuminated by a light, set on the lid, the other compartment is dark and without illumination. The two compartments communicate through a sliding-door system and the floor is a steel grid in both compartments. The bars of the dark compartment floor are wired to a constant current scrambler circuit [20]. The experimental session was divided into three phases: habituation trial, acquisition trial and retention trial [21]. During the habituation trial, the gerbil is placed in the white and illuminated compartment. In this phase, the sliding doors are initially closed and they open after 3 s. The gerbil can now explore both compartments for 90 s and, after this period, it is taken off the

apparatus. After 12 min, it is placed again in the white compartment. The sliding doors open after 3 s and successively close when the gerbil crosses the cage, entering the dark room, where it remains for 10 s, then is removed from the cage. The acquisition trial is performed 60 min after the habituation trial. In this phase, the gerbil is replaced in the white room and when it crosses the sliding doors entering the dark room, it receives an electric shock (4 mA for 5 s), released from the grid. Twenty-four hours later, the retention trial is performed. The gerbil is replaced in the white room and the sliding doors open as in the previous phases after 3 s. During this phase a timer measures the response latency as the period, in seconds, between the time when the gerbil is placed in the white room and the moment when the animal

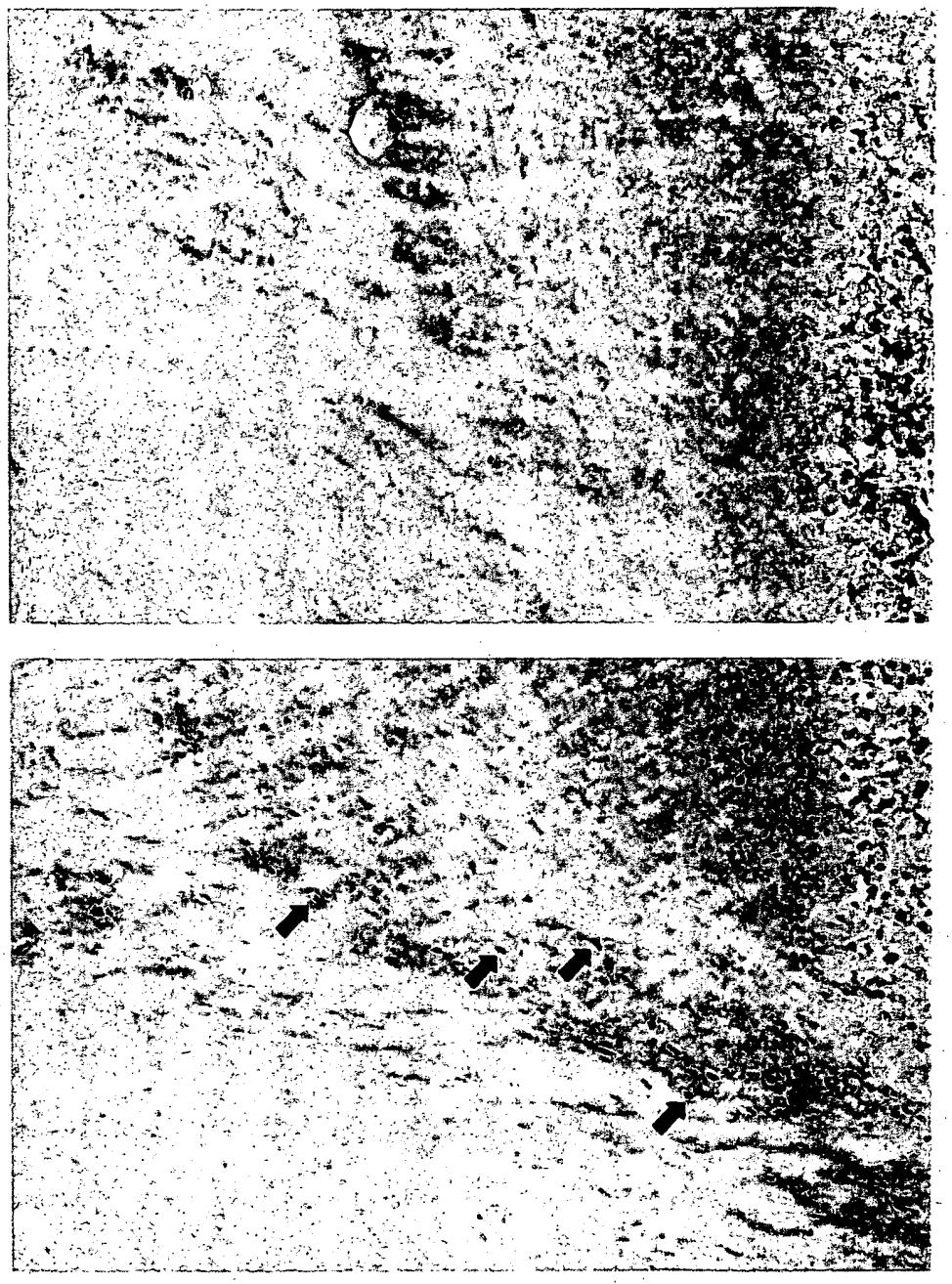
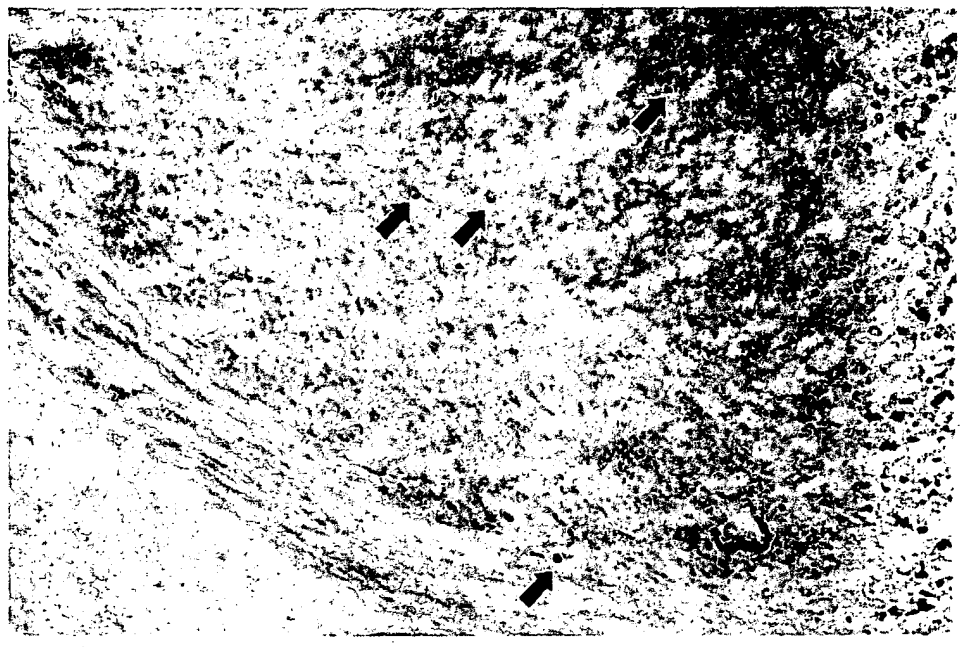


Fig. 1. Immunohistochemical observation of sections of (A) sham animals treated with rAAV-LacZ 12 days before the surgery, (B) animals subjected to transient brain ischemia, (C) sham animals treated with rAAV-VEGF 12 days before surgery. (A) Absence of immunopositive VEGF-elements in brain coronal section of thalamus of sham-operated animals treated with rAAV-LacZ (×10). (B) Several VEGF-immunostained neurons in a section of animals subjected to transient brain ischemia (×10). (C) Immunostaining for VEGF in sections of reticular nucleus of thalamus of animals sacrificed 12 days after i.c.v. rAAV-VEGF (×10). Each column represents the mean ± S.E. of six animals. $^{*}P < 0.05$ vs. ischemia.



(C)

Fig. 1. (*Continued*).

crosses in the dark compartment. The cut-off time was set at 300 s.

3.4. Statistical analysis

All statistical procedures were performed using SPSS statistical software package release 6.1.3 (SPSS Inc., Chicago, IL, USA). Data analysis was performed using one-way analysis of variance (ANOVA) with Scheffè's post hoc test for multiple comparisons. Each value represents the mean ± S.E.M. of six animals. Statistical significance was assumed for $P < 0.05$.

4. Results

4.1. Immunohistochemistry

Immunohistochemical evaluation of brain coronal sections of control animals treated with rAAV-LacZ showed absence of immunopositive VEGF-elements in all brain regions of the sections studied including the thalamus (Figs. 1A and 2). In animals subjected to transient brain ischemia several neurons within the thalamic reticular nucleus displayed VEGF-immunostaining (Fig. 1B and 2). In sections of animals sacrificed 12 days after i.c.v. rAAV-VEGF injection, immunostaining of reticular nucleus is evident but lesser with respect to ischemic animals (Fig. 1C and 2). There was not a significant difference between animals subjected to rAAV-VEGF injection 6 days versus 12 days before ischemia (data not presented).

4.2. Survival and brain edema

Treatment with the rAAV-VEGF vector significantly increased survival in the first 24 h of animals subjected to transient brain ischemia, either when injected 6 (+12.5%) or 12 days (+16.7%) before ischemia, with respect to animals treated with rAAV-LacZ reporter gene (control gene) (Table 1). Assessment of post-ischemic tissue water content revealed that rAAV-VEGF injected 6 and 12 days before ischemia produced a significant reduction of post-ischemic brain edema when compared to water content of LacZ reporter gene treated animals (Fig. 3).

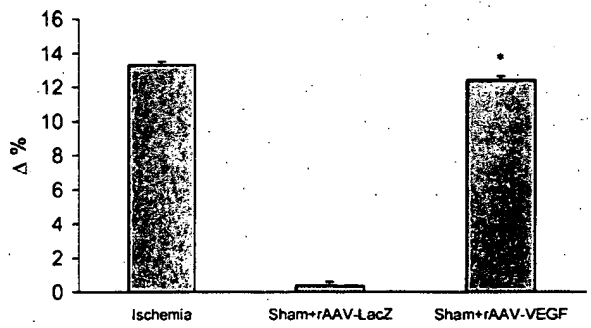


Fig. 2. Immunohistochemical observation of coronal sections of thalamus of animals subjected to: (i) transient brain ischemia, (ii) sham-operated and treated with with rAAV-Lacz 12 days before surgery, (iii) sham-operated and treated with rAAV-VEGF 12 days before surgery. Each column represents the mean ± S.E. of five to six animals. * $P < 0.01$ vs. sham + rAAV-LacZ.

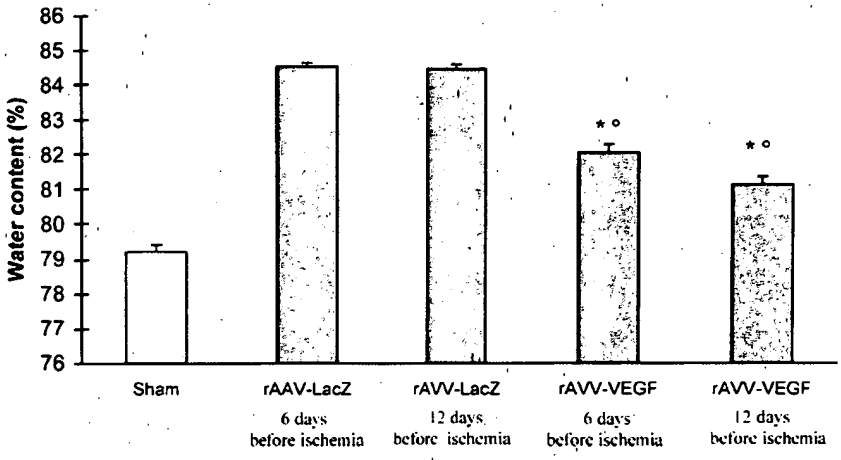


Fig. 3. Effects of rAAV-VEGF or rAAV-LacZ administration 6 or 12 days before ischemia on post-ischemic brain edema evaluated through water content in the hippocampus. Each column represents the mean ± S.E. of five to six animals. * $P < 0.001$ vs. sham; ° $P < 0.001$ vs. rAAV-LacZ.

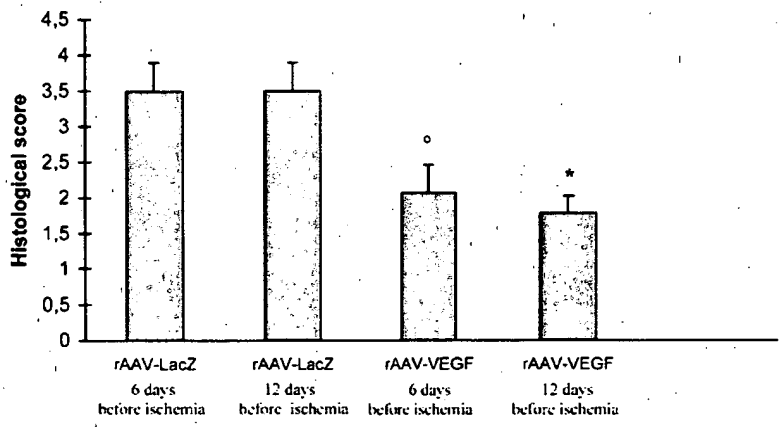


Fig. 4. Effects of rAAV-VEGF or rAAV-LacZ administration 6 days or 12 days before ischemia on post-ischemic delayed neuronal death in the CA1 area after transient cerebral ischemia. Each column represents the mean ± S.E. of five to six animals. * $P < 0.01$ vs. rAAV-LacZ; ° $P < 0.05$ vs. rAAV-LacZ.

4.3. Delayed neuronal death

Histological observation of delayed neuronal death localized in the CA1 of animals subjected to transient brain ischemia was significantly attenuated in animals treated with rAAV-VEGF injection either 6 or 12 days before with respect to those treated with rAAV-LacZ control gene (Fig. 4).

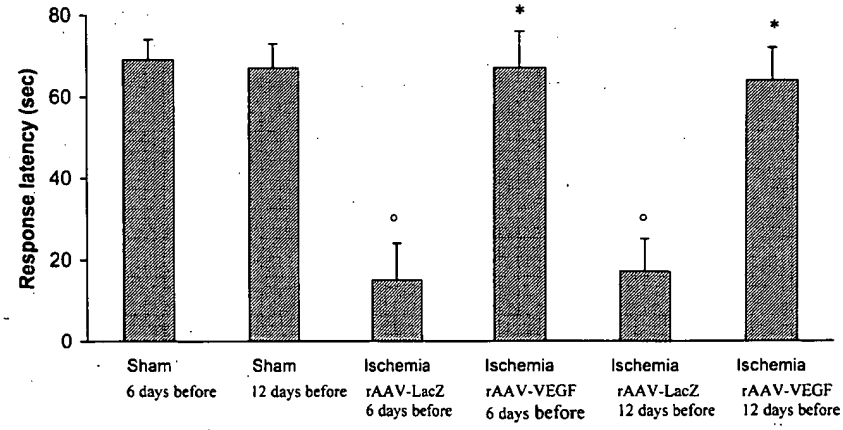


Fig. 5. Effects of rAAV-VEGF or rAAV-LacZ injection 6 and 12 days before ischemia on learning ability after ischemia or sham-operation evaluated through a passive avoidance apparatus. Each column represents the mean ± S.E. of five to six animals. ° $P < 0.01$ vs. sham; * $P < 0.01$ vs. rAAV-LacZ.

4.4. Passive avoidance

At the passive avoidance test, the gerbils subjected to brain ischemia and treated with rAAV-LacZ, showed a reduced response latency when compared to that of sham animals (Fig. 5). These data indicate that the ischemia reduced the ability of learning the information provided during the test. In animals treated with rAAV-VEGF, response latency at the passive avoidance was significantly improved with respect to animals subjected to ischemia and it was similar to that of sham-animals (Fig. 5).

5. Discussion

VEGF mRNA is widely expressed in the adult rat brain, mainly in the epithelial cells of the choroid plexus but also in astrocytes and neurons [22]. VEGF is involved in the physiopathology of cerebral ischemia. Hypoxia induced by focal brain ischemia represents a known stimulus for expression of VEGF [23] and of its receptor (VEGFR) [24]. Studies conducted on expression of VEGF/VEGFR strongly suggest that post-ischemic activation of this system leads to the growth of new vessels after cerebral ischemia [25]. It has been also reported that VEGF itself, when topically applied to the surface of a reperfused rat brain after transient cerebral artery occlusion, reduces ischemic brain injury [26].

An increase in VEGP expression was seen in the ischemic region and was found to be localized in most cell types such as neurons and glia, as well as infiltrating macrophages. This enhancement of VEGF expression has been linked with cells associated to CNS inflammation including microglial cells, and reactive astrocytes, thus indicating a role of VEGF in CNS inflammation [27].

The temporal profile of expression of VEGF and its receptors has been studied in different animal models of brain ischemia and is variable depending on the experimental model used. In general, increased expression of VEGF and its receptors occurs several hours following brain ischemia reaching its maximum after 1–2 days after the onset of the hypoxic insult [28] and then being maintained for several days [24].

The role of VEGF in ischemic and hypoxic conditions has been confirmed by experiments performed in vitro. It has been observed that VEGF protects primary cultures of rat cortical neurons from hypoxia and glucose deprivation [13].

Techniques for inserting DNA into cells have been investigated for treating various conditions affecting CNS. DNA transfer may be achieved using AAV vectors. Adeno-associated viruses are DNA viruses non-pathogenic to humans and rodents and integrate into the host genome [29]. After cerebral ischemia these vectors may be used to upregulate genes coding for protective proteins exerting beneficial effects on brain ischemia injury [30,31]. Genetic vectors cannot cross the intact blood brain barrier and therefore must be injected directly into the CNS the cerebral ventricles [32]; alternatively these can be stereotactically inoculated into the brain parenchyma [33].

After adenovirus-mediated gene transfer, peak expression is observed within a week and diminishes by 3 weeks [34]. In the present study we investigated the effects of AAV carrying the gene promoting VEGF expression 6 and 12 days before global cerebral ischemia.

Following rAAV-VEGF injection in the lateral cerebral ventricles, induction of VEGF expression was present 6 days and 12 days after the administration. Moreover, we observed that when rAAV-VEGF was injected in animals subjected to transient brain ischemia, expression of VEGF was significantly enhanced with respect to ischemic animals in which rAAV-LacZ (control gene) was injected.

Immunohistochemistry revealed, that post-ischemic VEGF expression was limited to the diencephalic region, in particular to the reticular thalamic nucleus. The thalamus is one of the most vulnerable sites to ischemia induced by carotid occlusion, and thalamic damage seems to be related to accumulation of calcium granules and an active process of calcification [35]. Also, immunostaining in tissue slices of animals receiving rAAV-VEGF and not subjected to ischemia (controls) revealed increased expression of VEGF within the thalamus.

Expression of VEGF remote from the site of injection evident in our experiments may be explained (a) by vector-mediated expression of VEGF within the ependymal cells lining the ventricular system and (b) its secretion into the cerebrospinal fluid as it has been hypothesized in similar experiments investigating leptin expression in brain tissue following gene therapy [36].

Our data indicate pre-ischemic intracerebroventricular application of rAAV-VEGF increases survival rate and reduces brain edema following transient global ischemia in the gerbil. VEGF modulates the permeability of microvessels apparently independent from changes in local hemodynamics induced by VEGF itself [37]. The exact mechanisms through which VEGF reduces edema and tissue damage after ischemia/reperfusion injury remain unclear. However, it has been hypothesized that neuroprotection could be mediated directly by changes in vascular water flux across the blood brain barrier [38].

There is evidence both in vivo and in vitro for VEGF playing a key role in cell survival [39]. Consistent with cellular pro-survival activity, VEGF induces expression of anti-apopoptic proteins such as Bcl-2. It has been also demonstrated that the pro-survival activity of VEGF is mediated by the phosphatidyl inositol (PI) 3′ kinase/Akt pathway [40,41]. VEGF delivered by adeno-associated vector may protect neurons from delayed neuronal death [42] by inhibition of apoptosis.

Interestingly, intracerebral injection of AAV vector produces enhancement of VEGF in the thalamus, one of the most vulnerable region to ischemia [43]. The thalamus, together with the hippocampus, has been shown to be involved in memory processes. It has been shown that lesions

within these regions in laboratory animals [44] and hypoxic-ischemic injury in humans involving reduction of gray matter in the thalamus [45,46] provoke deficits in learning and memory.

Inefficient vascular supply and the resultant reduction in tissue oxygen tension lead to the neovascularization mediated by VEGF [47]. Intraventricular rAAV-VEGF application produced expression of VEGF almost selectively in the thalamus. It therefore appears reasonable to hypothesize, that this brain region plays a key role in the neuroprotection produced by VEGF.

In conclusion, notwithstanding and clinical limitations of the study are existing regarding the time of pre-ischemic treatment, our work confirm that pre-ischemic gene therapy involving enhancement of VEGF in the brain exerts neuroprotective effects associated with prevention of learning impairment resulting from transient global cerebral ischemia and indicates that these effects could be related to increased expression of VEGF within the thalamus.

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Effect of adventitial $VEGF_{165}$ gene transfer on vascular thickening after coronary artery balloon injury

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Abstract

Objective: Experimental studies have provided evidence that neovascularization is an important feature of plaque growth, and angiogenic gene therapy may, therefore, increase plaque growth. This study examined the effect of local (peri)adventitial vascular endothelial growth factor165 (VEGF) gene transfer on vascular thickening after coronary balloon injury. **Methods**: Two coronary arteries of 15 pigs were subjected to balloon injury followed by either (peri)adventitial VEGF165 or β-galactosidase (LacZ) plasmid/liposome-mediated gene transfer via needle injection catheter. At days 3, 14 and 28, histologic sections of coronary arteries were analyzed. **Results**: Transferred VEGF165 gene and increased adventitial neovascularization were detected in coronary arteries after balloon injury and VEGF injection. The mean intima + media (I + M) area increased after coronary balloon injury and VEGF (1.13 ± 0.17 and 2.54 ± 0.52 mm^2) or LacZ (1.37 ± 0.19 and 2.96 ± 0.41 mm^2) gene transfer, with no significant difference between both groups at 3 and 28 days, respectively. No significant difference in I + M neovascularization was observed at day 28 between the treatment groups (microvessel area density $0.24 \pm 0.08\%$ with VEGF and $0.26 \pm 0.14\%$ with LacZ, respectively). I + M endothelial cell proliferation index ranged from 7% to 22% (VEGF) and 18% to 24% (LacZ). **Conclusions**: Catheter-mediated (peri)adventitial VEGF165 gene transfer induces adventitial neovascularization but not an increase of vascular thickening/I + M growth and vascularization in a porcine model of coronary artery injury.

Keywords: Angiogenesis; Atherosclerosis; Gene therapy; Growth factors

1. Introduction

Vascular endothelial growth factor (VEGF) is a proangiogenic cytokine essential for vasculogenesis and postnatal angiogenesis, including cardiovascular diseases [1,2]. VEGF has been used in clinical studies to stimulate collateral vessel formation for the treatment of ischemic heart disease, denoted therapeutic angiogenesis [3]. In spite of the potential beneficial therapeutic effect of VEGF, experimental studies have raised concerns about the safety of VEGF-mediated angiogenesis. These studies have shown evidence that plaque/lesion neovascularization is necessary for plaque growth [4,5]. A recent study by Celletti et al. [6] addressed the question whether angiogenesis is not only necessary but sufficient for plaque growth. They found that a single intramuscular injection of recombinant human VEGF protein in a hypercholesterolemic rabbit model results in an increase in aortic plaque size. However, as we have only begun to understand new facets in the complexity of VEGF biology, it is unknown if these results can be transferred to the setting of local VEGF gene therapy targeting the cardio-

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vascular system. Moreover, the clinical relevance of the reported data has to be elucidated as experimental and clinical studies suggest that local therapy may be superior over systemic therapy and gene therapy over administration of recombinant proteins [7,8]. There is still no evidence for lesion acceleration after human coronary/myocardial or peripheral angiogenic gene therapy [9–11]. We, therefore, examined the effect of local (peri)adventitial $VEGF_{165}$ gene transfer, comparable to the therapeutic modality used in primarily successful human angiogenic gene therapy studies, after mechanical coronary lesion induction on intima + media (I + M) microvessel (MV) angiogenesis and vascular thickening/I + M growth [12].

2. Methods

2.1. Animal model

All studies were carried out with approval of the Animal Care Committee of the State Office Berlin and in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996). Fifteen domestic, juvenile, crossbred swine were fed a standard pig chow without cholesterol supplementation. One hour prior to surgery, each animal was sedated with ketamine (25 mg/kg) intramuscularly, and ketamine (25 mg/kg) and xylazine (83 mg/kg) were given intravenously for general anesthesia. Atropine (1 mg) was administered intramuscularly to reduce orotracheal secretions. Throughout the procedure, the electrocardiogram and arterial blood pressure were monitored. Arterial access was obtained via percutaneous puncture of the femoral artery and an 8-french arterial sheath was inserted.

Amiodarone (5 mg/kg) was given prophylactically to prevent ventricular arrhythmias. A heparin bolus (5000 units) was given intravenously. Two of the three major coronary arteries of each animal were subjected in a randomized fashion to balloon injury followed by periadventitial/perivascular injection of either therapeutic ($VEGF_{165}$) or control (β-galactosidase = LacZ; plasmid and liposomes are described subsequently) genes in the injured coronary artery segment using a needle injection catheter (Bavaria Medizin Technologie, Oberpfaffenhofen, Germany). The remaining coronary artery served as a noninjured control artery. This study design was chosen to validate the effect of VEGF gene transfer in comparison to an appropriate control using exactly the same procedure (LacZ group) and in comparison to an uninjured artery to show that the effect of VEGF gene transfer was limited to the target artery without systemic effects in the coronary circulation (three major coronary arteries of the same animal for comparison).

Coronary angioplasty was performed under fluoroscopic guidance in the proximal segment of one of the three main coronary arteries (e.g., left anterior descending, left circumflex and right coronary arteries) using standard guidewire and balloon technique with a standard balloon angioplasty catheter (diameter 4 mm; Medtronic, Düsseldorf, Germany), to ensure a balloon to artery ratio of 1.3–1.5:1. Coronary angiography was performed after administering 200 μg of intracoronary nitroglycerin before and after percutaneous transluminal coronary angioplasty (PTCA) using an ionic contrast media to confirm normal blood flow and vessel patency. The arterial sheath was then removed and manual compression over the puncture site applied until the arterial wound was closed. Anesthesia was terminated and the pigs were observed in the recovery room prior to returning to their stalls. To label proliferating cells, each pig received a 50 mg/kg intravenous dose of 5-bromo-2′-deoxyuridine (BrDU; Roche, Mannheim, Germany) 1 h prior to euthanasia under general anesthesia (five pigs per time point at days 3, 14 and 28 after PTCA and gene transfer) with a euthanasia solution T 61 (Intervet, Unterschleißheim, Germany).

2.2. Histopathologic processing

Porcine hearts were harvested immediately after sacrifice and perfused with Ringers' lactate at 100 mm Hg for 10 min via pressure tubing seated in the ascending aorta. This was followed by perfusion fixation with 10% neutral buffered formalin at 100 mm Hg for 60 min. Hearts were immersion-fixed in 10% neutral buffered formalin overnight, and processed the following day as previously described [13]. Briefly, 30 mm of artery from the proximal angioplasty sites for each coronary artery was dissected from the heart en bloc and embedded in paraffin for sectioning at 5-μm intervals. For each representative arterial segment, a minimum of eight serial sections were stained with hematoxylin and Elastica van Gieson for morphometric analysis as described below. A section of small bowel was resected from each pig and used as positive control tissue for BrDU immunolabeling.

2.3. Plasmid/liposomes

For in vivo gene transfer, the following plasmid and liposome complexes were made: 50 μg pCMV-$VEGF_{165}$ plasmid [containing the human VEGF cDNA (96% homologous with pig VEGF) nucleotides 57–629 in a pCIS expression vector, Genentech, South San Francisco, CA, USA] were slowly complexed with 50 μg of Lipofectine (Gibco-BRL, Grand Island, NY, USA) and diluted to a total volume of 600 μl with Ringer solution [14]. For transfection of dilated control arteries, a similar plasmid/liposome complex containing *Escherichia coli* LacZ cDNA CMV-expression plasmid (nucleotides 1–3100) was used [15]. The mixtures were kept at room temperature for at least 30 min and used for gene transfer within 2 h. Plasmids were isolated using Maxi columns (Qiagen, Hilden, Germany), purified using phenol/chloroform extractions and ethanol precipitation and analyzed for the absence of any microbiological or endotoxin contamination (Limulus assay; Sigma, St. Louis, MO, USA).

2.4. RT-PCR analysis

Twelve sections of paraffin-embedded tissue were deparaffinized with xylene and then washed with 100% ethanol. Total RNA was extracted using Rneasy Mini Kit columns (Qiagen) including DNase treatment. Furthermore, possible contamination with genomic DNA was excluded by control experiments omitting the reverse transcriptase. The samples were reverse-transcribed to the first-strand cDNA using Superscript II RT and random primers (Invitrogen, Karlsruhe, Germany). A 40-cycle PCR (cycle parameters: 30 s at 95 °C, 30 s at 62 °C, 30 s at 72 °C) was performed with Ampli Taq DNA Polymerase (Perkin Elmer Applied Biosystems, Foster City, CA, USA) and 100 μM primer pairs. Primers were designed to distinguish between endogenous porcine VEGF and transfected human $VEGF_{165}$ by selecting the antisense primer from the polyadenylation signal of the expression vector and the sense primer from the coding region. Primer sequences were used as follows: sense primer 5′-agcacggtccctcttggaat-3′ and antisense primer 5′-tgtaaccattataagctgcaataa-3′. To prove that the selected primers work reliably in the reverse-transcribed probes, in vitro experiments were performed by transfecting HaCat ceratinocytes with the same $VEGF_{165}$ plasmid used for in vivo gene transfer, then isolating total RNA and performing RT and PCR as described above.

2.5. Morphometric and microvessel analysis

Two independent investigators who were blinded to the nature of tissue sections (treatment) examined all slides. Morphometric analysis was performed on all arterial segments (minimum of eight sections per segment) and the cross section with the most severe luminal narrowing was identified for study analysis (including calculation of the injury score) using digitized images. The images were magnified in a Leica DMRD microscope (Leica, Bensheim, Germany) and captured by a Sony 3CCDTM (Charged Couple Device) true color (red-green-blue/RGB) video camera (Sony, Tokyo, Japan) mounted on the microscope by a Leica 0.5-in. C-mount adapter with 0.35 magnification level (Leica) and connected to a frame grabber. The grabbed images (704 × 548 pixel RGB format with a 24-bit resolution and 16 million colors) were digitized by a Matrox Comet 24-bit color graphic card. Images were analyzed employing the software LUCIA G (version 3.52ab, Nikon, Düsseldorf, Germany) in an Intel Pentium MMXTM 233-MHz-based personal computer and Microsoft. Because of interruptions of the internal elastic lamina post-balloon angioplasty, it was impossible to consider the intima and media as discrete entities, and therefore, a combined intima plus media (I+M) area was measured on Elastica van Gieson stained slides. I+M (= tissue area between the lumen area and the external elastic lamina) was defined as vascular thickening. To analyze I+M angiogenesis, the total number of MVs in the I+M area, as well as the percentage of I+M area occupied by MVs (MV area density) and MV size, was studied. Tissue slides of noninjured arteries without gene transfer, arteries after PTCA and VEGF or LacZ (control) gene transfer from all time points were immunolabeled with an anti-vWf antibody. Fifteen uninjured arteries and 30 balloon-injured arteries (15 with VEGF and 15 with LacZ gene transfer) were analyzed using the following protocol. All optical fields within the I+M area were evaluated by image analysis at 200 × magnification. In addition to counting the total number of MVs of all optical fields, the perimeter of MV within the I+M were traced and the MV area measured. The I+M microvascular area density was calculated for each artery as summed MV area of the I+M divided by the total I+M area (quotient expressed as a percentage by multiplying by 100). To determine the average MV size for a group of arteries, we divided the MV perimeter by the MV number to obtain the MV size index of each artery (expressed in μm). A large index indicates that the average microvessel size is large.

2.6. Endothelial cell proliferation

To quantify the levels of endothelial cell proliferation that accompany I+M MV angiogenesis, we quantitatively analyzed at 400 × magnification the total number of endothelial cells (ECs) and the total number of BrDU immunoreactive ECs on adjacent cross sections of the same artery. The proliferation index (percentage of BrDU immunoreactive ECs) was then calculated for the I+M of each artery.

2.7. Immunohistochemistry

Immunohistochemical labeling was carried out on adjacent tissue sections using previously described methods [16]. Five-micrometer tissue sections were deparaffinized using Roti-Histol (Carl Roth, Karlsruhe, Germany) and then rehydrated in a decreasing ethanol series.

2.7.1. Staining of ECs

After pretreatment with 0.25% pepsin–HCl solution, slides were incubated with a 1:250 rabbit anti-vWf polyclonal antibody (DAKO Diagnostika, Hamburg, Germany) for 45 min at 37 °C. A biotinylated swine antirabbit antibody was applied for 45 min followed by an streptavidin/biotinylated alkaline phosphatase complex (both from DAKO Diagnostika) for 30 min. Alkaline phosphatase activity was visualized with Naphthol AS-MX Phosphate (Fast Red, DAKO Diagnostika).

2.7.2. Staining of replicating cells

After a 5-min incubation with 3% hydrogen peroxide and a 15-min pretreatment with HCl followed by a 7-min digest with 0.1% trypsin, slides were incubated with a 1:25 mouse anti-BrDU monoclonal antibody (Amersham Biosciences Europe, Freiburg, Germany) for 2 h at 37 °C. A ready-to-use biotinylated horse anti-rabbit/mouse antibody was applied for 30 min followed by a 30-min incubation with ABC

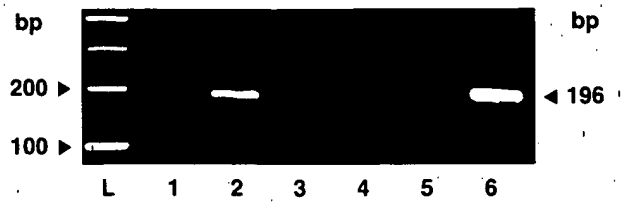

Fig. 1. VEGF transgene expression in porcine coronary arteries. Expression of the transferred VEGF cDNA in VEGF- and LacZ-transfected arteries 3 days after balloon injury and needle injection catheter delivery as analyzed using RT-PCR. Lane 1, Control PCR without previous reverse transcription; lane 2, VEGF-transfected coronary artery with an expected 196-bp amplified fragment indicating the expression of the transgene; lane 3, LacZ-transfected artery showing no transgene expression; lane 4, same as lane 2 but 5′ primer omitted; lane 5, same as lane 2 but 3′ primer omitted; lane 6, positive control plasmid for VEGF transgene; lane L, DNA size markers using a 100-bp ladder.

Reagent (RTU Vectastain universal Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA). Horseradish peroxidase activity was visualized with 3-amino-9-ethyl carbazole (AEC Substrate Kit, DAKO Diagnostika). Porcine small bowel served as positive control tissue. Incubations with PBS containing 1% BSA and isotype-matched immunoglobulins were used as negative controls for all immunostainings. Hematoxylin was used as nuclear counterstain.

2.8. Statistical analysis

Quantitative data were expressed as mean ± S.E.M. For comparison of multigroup variables (for each group at each time point), the variance of means was analyzed using a one-way ANOVA. If the *F*-test results were significant, post hoc comparisons were carried out using a Fisher LSD test to perform multiple pairwise comparisons among and an unpaired *t*-test was used to compare the treatment groups. Statistical significance was defined by a p value < 0.05.

3. Results

3.1. VEGF transgene expression

Expression of the transfected $VEGF_{165}$ was demonstrated at 3 days after PTCA and VEGF gene transfer by RT-PCR using primers specific for the transgene. Transgene expression was not found in coronary arteries after PTCA and LacZ gene transfer (Fig. 1).

Table 1
Injury score

	Number of vessels	Injury score
VEGF	15	1.77 ± 0.530
LacZ	15	1.80 ± 0.414

Injury score expressed as extend of fragmentation of IEL, media and EEL at sites of maximal injury in coronary arteries in VEGF- and LacZ-treated groups: 0.5 points for minimally disrupted IEL + intact media and EEL; 1.0 points for lacerated IEL + intact media and EEL; 1.5 points for lacerated IEL + less than half thickness of media lacerated + intact EEL; 2.0 points for lacerated IEL + more than half thickness of media lacerated + intact EEL; 2.5 points for lacerated IEL + media totally lacerated + EEL minimally disrupted [32]. Data are shown as means ± S.D.

3.2. Injury score

Table 1 shows the mean injury score of the evaluated arterial segments. Noninjured control arteries did not show any fragmentation of the arterial wall. The severity of injury to coronary arteries was similar in both VEGF- and LacZ-treated groups.

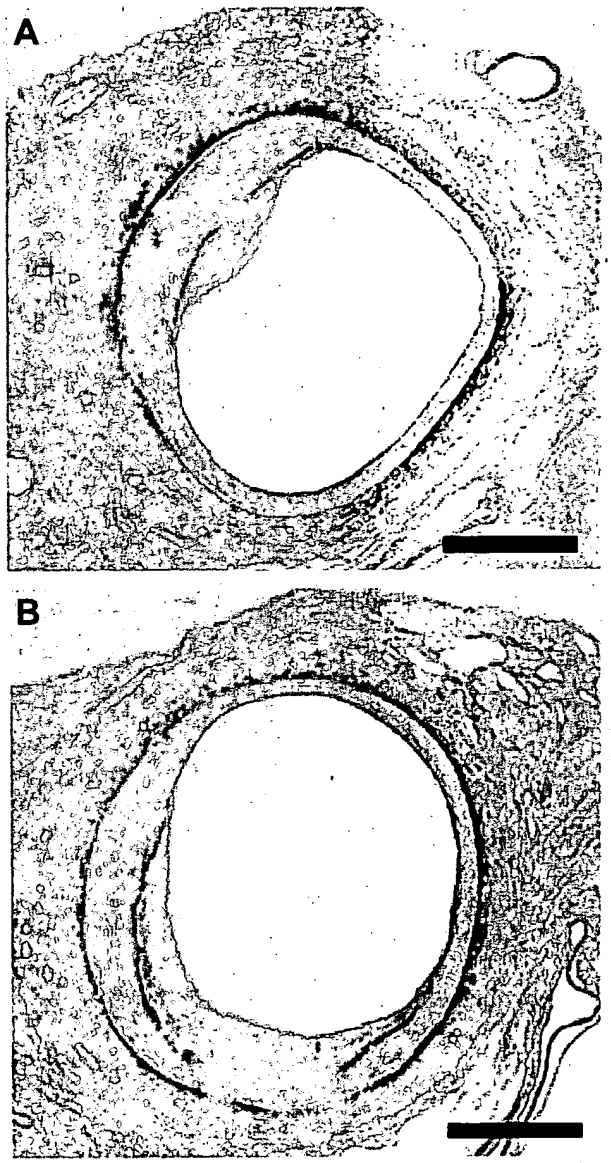

Fig. 2. Histomorphology of VEGF- and LacZ-transfected coronary arteries. Representative photomicrographs 28 days after coronary angioplasty and VEGF (A) or LacZ (B) gene transfer using the needle injection catheter showing the intima plus media lesion development/vascular thickening. Both slides were stained with Elastica van Gieson (bar = 1 mm).

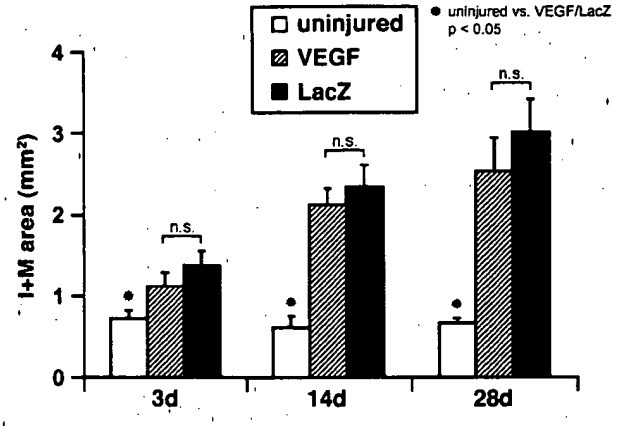

Fig. 3. Intima + media area/vascular thickening. I + M area increased significantly with time after balloon injury in both treatment groups (VEGF = hatched bars; LacZ = black bars) in comparison to uninjured arteries (open bars). There was a statistically significant difference between uninjured and both injured VEGF and LacZ transfected arteries at all time points studied (*$p < 0.05$). In contrast, there was no significant difference in the I + M area/vascular thickening until day 28 after intervention and gene transfer between VEGF- and LacZ-transfected arteries.

3.3. Vascular thickening/intima plus media area

Fig. 2 shows representative photomicrographs of coronary arteries 28 days after PTCA plus VEGF (Fig. 2A) or PTCA plus LacZ (Fig. 2B) gene transfer. As detailed in Fig. 3, the mean I + M area is significantly increased with time after PTCA in both VEGF- and LacZ-treated groups compared with noninjured arteries without gene transfer. No statistically significant difference was observed between the VEGF- and LacZ-treated groups at day 28 after PTCA (2.54 ± 0.52 mm^2 with VEGF vs. 2.96 ± 0.41 mm^2 with LacZ, $p = 0.61$, respectively).

3.4. Microvessel angiogenesis

3.4.1. Microvessel number

Fig. 4 shows magnifications of the coronary arteries illustrated in Fig. 2 of the vascular thickening/I + M growth with I + M microvessel angiogenesis (EC immunohistochemical staining with vWf-antibody) 28 days after PTCA plus VEGF (Fig. 4A) or PTCA plus LacZ (Fig. 4C) gene

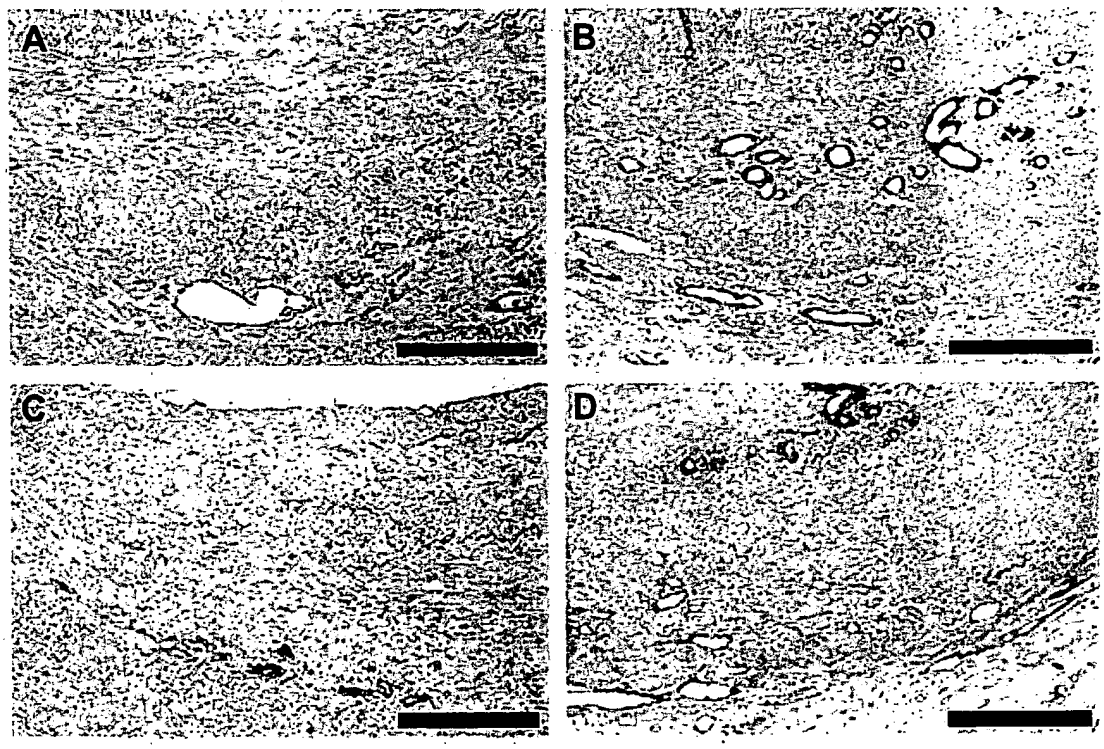

Fig. 4. Intima + media and adventitia microvessel angiogenesis. Photomicrographs of microvessel angiogenesis 28 days after coronary angioplasty and VEGF or LacZ gene transfer with the needle injection catheter. Adjacent cross sections of the coronary arteries shown in Fig. 2 immunolabeled with an antibody to vWf showing ECs (endothelial cells) of intima + media (A) and adventitia (B) microvessels from VEGF-transfected artery and ECs of intima + media (C) and adventitia (D) microvessels from LacZ-transfected artery. Note the significant microvessel angiogenesis in the adventitia of VEGF-transfected compared to LacZ-transfected arteries and the similar angiogenic microvessel response in the intima + media area after (peri)adventitial VEGF and LacZ plasmid/liposome transfer, respectively (bar = 200 μm).

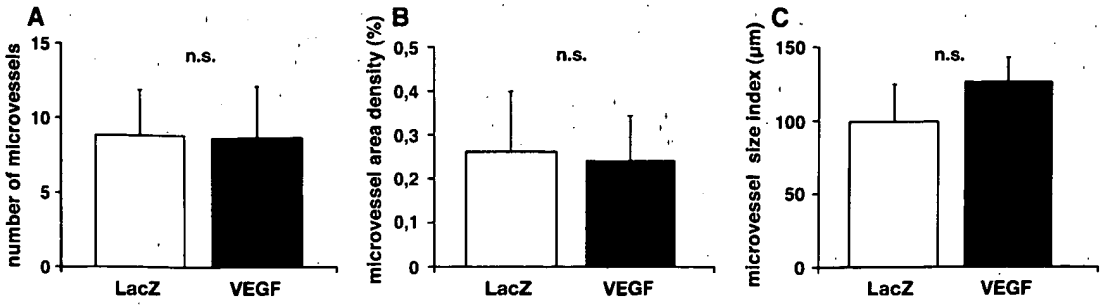

Fig. 5. Intima + media microvessel image analysis. I + M angiogenesis was absent until day 14 after balloon injury and VEGF or LacZ gene transfer and only occurred reproducibly 28 days after intervention. There was no significant difference in microvessel number (A) and microvessel area density (B) in the I + M area between VEGF (black bars) and LacZ (open bars) transfected arteries at 28 days after balloon injury. The microvessel size index of the I + M microvessels 28 days after intervention (C), reflecting not only angiogenic but also potential vasodilatory effects was similar in the VEGF- and LacZ-treated groups.

transfer and of the adventitia with adventitial microvessel angiogenesis (EC immunohistochemical staining with vWf-antibody) of VEGF- (Fig. 4B) and LacZ- (Fig. 4D) treated coronary arteries. These figures demonstrate that there was a pronounced adventitial microvessel angiogenesis in the VEGF gene therapy arm compared to the LacZ gene transfer (control) arm 28 days after intervention. In contrast, histologically, no difference was observed with respect to I + M microvessel angiogenesis at day 28 after PTCA in both treatment groups. Image analysis of MVs in the I + M revealed no statistical significant difference in MV number between the VEGF and control gene (LacZ) treated coronary arteries (8.67 ± 3.48 for VEGF vs. 8.80 ± 3.10 for LacZ, $p = 0.97$ respectively; Fig. 5A) at the only time point after intervention (28 days) when I + M microvessel angiogenesis occurred regularly. I + M MV angiogenesis was absent until day 14 and only observed in two animals of each treatment group at day 14 after intervention.

3.4.2. Microvessel area density and microvessel size index

As MV number may not reflect the relative I + M vascularity in relation to an increased I + M area over the time course after PTCA, we analyzed the I + M area comprised of MVs (= MV area density). Further, since changes in the MV area density are not only due to alterations in microvessel number but also due to changes in the microvessel size, we therefore calculated a microvessel size index. Similar to MV number, there was no difference in MV area density ($0.24 \pm 0.08\%$ for VEGF vs. $0.26 \pm 0.14\%$ for LacZ, $p = 0.91$, respectively) or MV size index (126 ± 16 µm for VEGF vs. 98 ± 25 µm for LacZ, $p = 0.36$, respectively) between both treatment groups 28 days after PTCA (Fig. 5B,C).

3.4.3. Endothelial cell proliferation

One important feature of angiogenesis is the EC proliferation. In all coronary arteries where I + M MV angiogen-

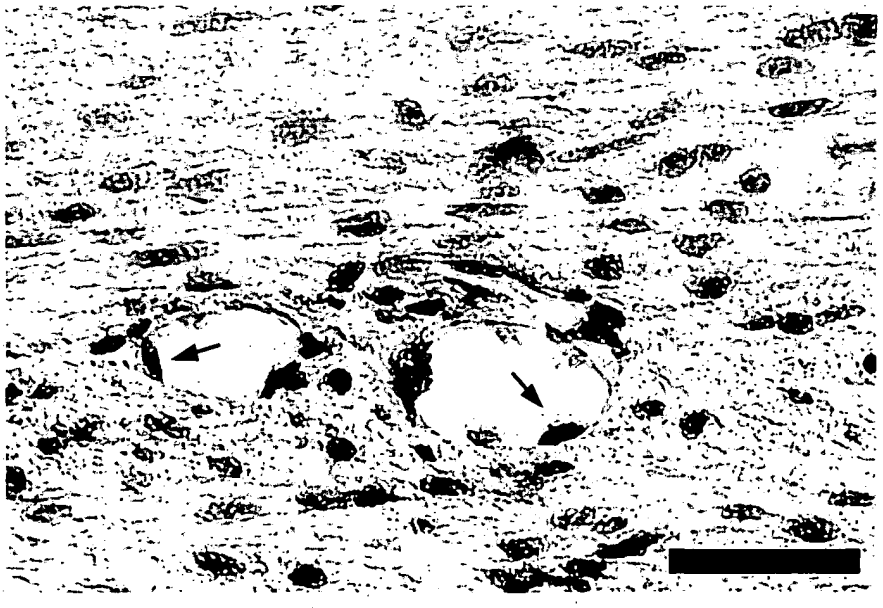

Fig. 6. Endothelial cell proliferation of intima + media microvessels: Immunostaining for BrdU of a histological cross section of a coronary artery 28 days after coronary angioplasty and VEGF gene transfer demonstrating proliferating endothelial cells (arrows) of microvessels in the I + M (bar = 40 µm).

esis occurred, we analyzed the total number of ECs (endothelial staining with vWf antibody), the total number of BrdU immunopositive (=proliferating) ECs as well as the resulting EC proliferation index. Proliferation of microvessel ECs in the I+M (Fig. 6) was only observed in one LacZ-treated animal 14 days after intervention (EC proliferation index 9%) and in two VEGF-treated animals, and three LacZ-treated animals 28 days after intervention with an EC proliferation index ranging from 7% to 25% (7–22% for VEGF and 18–24% for LacZ).

4. Discussion

VEGF gene therapy is a promising approach to induce therapeutic angiogenesis for the treatment of ischemic myocardial disease [3]. Recent experimental studies have shown that plaque neovascularization is necessary for plaque growth, and that systemic application of VEGF protein enhances atherosclerotic plaque progression [5,7,17]. Despite the ample evidence that VEGF functions as a vascular protective factor, the conflicting data from these experimental studies have raised concerns about the safety of VEGF angiogenic therapy and its potential detrimental effect via modulation of plaque microvessel angiogenesis in atherosclerosis [18,19]. The harmful effects of VEGF therapy were shown in small animal models looking at peripheral arteries or aortas and using a systemic approach of VEGF protein application. We therefore examined the effect of local (peri)adventitial VEGF gene delivery on vascular thickening/I+M area and I+M vascularity in porcine coronary arteries, resembling more closely the characteristics of the coronary/myocardial VEGF gene therapy applied in clinical studies [3]. For catheter-based local gene delivery, the needle injection catheter was used in this study. In contrast to the majority of balloon catheter delivery systems, the needle injection catheter allows an active delivery with high efficiency, providing a peri(adventitial) gene depot without causing a vessel wall trauma due to its ultrathin needles [20,21].

Using the balloon injury model to induce lesion development, our study showed that local (peri)adventitial delivery of $VEGF_{165}$ DNA induces a biological effect and results in adventitial neovascularization but does not increase I+M neovascularization nor vascular thickening/I+M growth.

4.1. VEGF and lesion neovascularization and vascular thickening

Reduction in aortic plaque growth in apolipoprotein-E-deficient mice by antiangiogenic therapy with inhibition of intimal neovascularization suggests an important role of plaque or lesion microvascularity in plaque/lesion development [5]. However, the role of VEGF in lesion development, possibly promoting plaque vascularization as a potential prerequisite for plaque expansion, was not investigated. Recent studies by Celletti et al. [6,17] gave insight into the potential role of VEGF in this pathophysiological process, and showed that VEGF administration by itself stimulates plaque vascularity and growth. The present study did not show an increase in I+M lesion microvascularity and vascular thickening/I+M growth after (peri)adventitial $VEGF_{165}$ in comparison to control (LacZ) gene administration post coronary balloon injury. In context with these different observations, it is important to note that in the above-mentioned studies, VEGF has been given systemically, inducing an increase in bone marrow and blood macrophages/monocytes. Macrophages modulate lesion formation and atherogenesis due to the induction of growth factors (prothrombotic, proliferative and angiogenic) and cytokines [22,23]. Therefore, it is possible that the observed increase in plaque vascularity after systemic VEGF administration results from systemic VEGF-mediated macrophage recruitment and not from direct local VEGF effects on the vascular wall. As local arterial wall macrophage accumulation was only observed 3 weeks after systemic VEGF administration (despite an early macrophage increase in bone marrow and blood), it seems unlikely that VEGF-induced macrophage accumulation in the plaque would also occur, at least to the same degree after local VEGF gene transfer. In addition to species differences and the variable response to injury of coronary and peripheral arteries or aortas (chronic atherosclerotic and acute mechanical vessel wall reaction), this may be an important reason for the difference in plaque/I+M lesion vascularity seen in our data compared to the other studies. In accordance with this, local perivascular VEGF gene transfer using the collar model in rabbits resulted in neovascularization only in the adventitia and not in the neointima, whose area was reduced 14 days after gene transfer [24]. Our study showed similar results with increased neovascularization only in the adventitia but not in the I+M lesion, and no subsequent increase of the vascular thickening/I+M growth 14 and 28 days after (peri)adventitial VEGF gene transfer. The fact that we did not see a reduction in vascular thickening may be explained by the difference in lesion characteristics and severity induced by balloon injury compared to the silastic collar as well as by the differences in species and arteries.

4.2. VEGF and spatial patterns of microvessel angiogenesis

Despite a pronounced angiogenesis of adventitial microvessels, no increase in I+M microvascularity was observed in our study. The adventitial angiogenesis was paralleled by VEGF mRNA expression in the outer compartment of the adventitia detected by in situ hybridization, whereas no VEGF mRNA expression was found in I+M 14 days after balloon injury and VEGF gene transfer (data not shown). The spatial patterns of vessel wall neovascularization and various parameters evaluating the changes in I+M microvascularity (MV number, size and area density and EC

proliferation) have been studied intensively and raise the question concerning a potential different biological role of adventitial and I+M vascularization in lesion development. It has not been investigated in the VEGF studies showing plaque acceleration where increased vascularity was located (at luminal surface, in the plaque or adventitia). In previous studies with PTCA of porcine coronary arteries, it has been shown that regression of adventitial MVs corresponds with arterial narrowing and an inverse correlation of adventitial MV area density and neointimal hyperplasia despite a positive correlation of MV number and neointimal increase was observed [16,25]. These results suggest a beneficial effect of the adventitial microvascularization and emphasize the importance of analyzing the percentage of vessel wall area covered by MVs rather than solely the MV number to assess the effect of MV neovascularization. Our study did not show any changes neither in I+M MV number nor in MV area density after PTCA and peri(adventitial) VEGF in comparison to control (LacZ) gene transfer, suggesting that local delivery of the $VEGF_{165}$ gene to the outer compartments of coronary arteries does not cause acceleration of lesion formation.

4.3. VEGF therapy regimen in clinical and experimental studies

The first Phase I clinical trial with local intramyocardial VEGF gene delivery showed a reduction in angina and an increase in cardiac perfusion in a small number of patients [12]. These promising results could not be confirmed in the randomized Phase II VIVA trial but VEGF delivery was intracoronary (not intramural but intraluminal) with the unavoidable flush away effect with blood flow. Besides, VEGF protein, and not cDNA, was used in this study [26]. Recent Phase I and I/II clinical trials used a catheter-mediated intramyocardial VEGF gene transfer and showed again a favorable outcome with reduced angina and improved myocardial perfusion in a higher number of patients [27,28]. Moreover, these trials and a Phase I study using a local catheter-mediated intracoronary (intramural) VEGF gene transfer did not show any evidence that atherosclerosis is accelerated after local gene transfer [10]. Despite these promising results, the discussion about the safety of VEGF gene transfer for therapeutic angiogenesis remains controversial. The critical question is, how experimental data, showing reduced plaque size in peripheral or aortic vessels after antiangiogenic therapy in hypercholesterolemic animal models and increased plaque size after VEGF therapy, relate to the above cited clinical data. In contrast to the successful clinical trials, angiogenic VEGF or antiangiogenic therapy in the experimental models was performed using a systemic delivery of proteins and the effect was not investigated in coronary arteries. Our experiments focused on the local delivery of VEGF gene into peri(adventitial) or perivascular tissue of coronary arteries and as reported in the clinical studies VEGF gene transfer did not show any evidence that lesion formation is accelerated after local gene transfer [11].

Another important issue in the evaluation of the safety of VEGF therapy is, apart from the delivery regimen (systemic versus local), the dosage of VEGF used for therapeutic angiogenesis. Unregulated/constitutive VEGF overexpression in a mouse model of myoblast-mediated VEGF gene delivery into the myocardium was associated with formation of intramyocardial vascular tumors and death [29]. These results emphasize that beneficial and harmful effects of VEGF therapy depend on the level of systemic and local VEGF concentration and underscore the importance of regulating VEGF expression for therapeutic angiogenesis. Taking this into consideration, it is important to know that low gene transfection efficiency and low local VEGF concentrations can achieve meaningful biological effects [30]. Therefore, in our study, a low dose of a single peri(adventitial) injection of 50 μg $VEGF_{165}$ plasmid was used. In comparison, in experimental and clinical studies without any observed side effects or lesion acceleration, 25–200 μg VEGF were locally delivered and levels of circulating angiogenic growth factors were either not measurable or in the picogram range [24,27,31].

4.4. Limitations

Even though the response to balloon injury of coronary arteries resembles many characteristics of the pathophysiology of coronary artery disease (e.g. smooth muscle cell/myofibroblast proliferation and migration, inflammatory reaction, intramural thrombus formation as seen in the instable plaque), the principal limitation of our study is that our porcine coronary arteries did not have a calcified, severely atherosclerotic plaque that may influence the biological effect of VEGF despite its local delivery away from the lesion/plaque.

5. Conclusions

Local delivery of low concentrations of VEGF gene into the outer compartment of coronary arteries induce adventitial angiogenesis but does not promote I+M microvessel angiogenesis nor accelerates vascular thickening/I+M growth in a porcine balloon injury model. This observation is consistent with results of clinical studies using the local gene transfer strategy for VEGF-induced therapeutic angiogenesis where no unwanted side effects (hemodynamically significant lesion acceleration) were observed. Our study is not in contradiction to the reports of earlier studies with an increase in plaque size after systemic VEGF protein application but underscores that vessel wall neovascularization is a finely tuned mechanism that depends on local VEGF concentration. Successful and safe VEGF therapy likely depends on local targeted delivery with a narrow therapeutic concentration range that differentiates the beneficial from

the negative VEGF effects. Long-term follow-up periods in clinical and experimental studies of local VEGF gene transfer are warranted to confirm the safety of VEGF therapy.

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Viral mediated gene transfer to sprouting blood vessels during angiogenesis

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Abstract

Several experimental systems have been applied to investigate the development of new blood vessels. Angiogenesis can be followed ex-vivo by culturing explants of rat aorta 'rings' in biomatrix gels. This angiogenesis system was modified for the study of viral vector mediated gene transfer, using adenovirus, vaccinia- and retroviral vectors. Two modifications were introduced to the model in order to facilitate efficient viral mediated gene transfer, (i) placing the aorta ring on top of a thin layer of collagen such that the angiogenic tissue will be accessible to the viral vector; and (ii) infection of the aorta rings prior to embedding them into the collagen matrix. While adenovirus and vaccinia vectors infected efficiently the aorta rings they induced cell death. Subsequent gene transfer experiments were, therefore, carried with retroviral vectors containing vascular endothelial growth factor (VEGF) and the β-interferon (IFN) genes. Overexpression of VEGF enhanced significantly microvessel sprouting, while overexpression of IFN-β induced an antiviral effect. The experimental system described in this study can facilitate the application of other viral vectors to the study of genes that may regulate the complex angiogenic process and thereby open new avenues for vascular gene therapy. © 2002 Published by Elsevier Science B.V.

Keywords: Viral vectors; Gene transfer; Angiogenesis; Aorta ring explant; Ex-vivo; VEGF

1. Introduction

Application of gene transfer methods to the study of angiogenesis and for the treatment of vascular diseases is in the forefront of recent cardiovascular research (Carmeliet, 2000; Freedman and Isner, 2001; Laham et al., 2001). Animal models and some clinical trials have indicated the potential of gene therapy in vascular diseases. In particular, the adenoviral vector and naked DNA

Abbreviations: BRDU, 5-bromo-2′-deoxyuridine; cfu, Colony forming unit; MuLV, moloney murine leukemia virus; pfu, Plaque forming unit; VEGF, vascular endothelial growth factor; VSV, vesicular stomatitis virus.

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[1] This work is dedicated in memory of Amiram Eldor, a talented physician, scientist, and a close friend who died tragically in an airplane accident.

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have been used extensively to deliver genes to the vasculature to prevent thrombosis and restenosis or to enhance angiogenesis (Indolfi et al., 1999; Hammond and McKirnan, 2001).

Angiogenesis, the development of new blood vessels from preexisting vasculature, is a multistep process that involves the intimal endothelium, smooth muscle cells, pericytes, fibroblasts, as well as extracellular matrix and soluble factors (Nicosia and Villaschi, 1999; Rosengart et al., 1999). Several experimental models have been developed to study this complex process and to dissect individual stages leading to the development of mature blood vessels. The animal models, while relevant to the human setting, do not allow study of the steps leading to the formation of a mature blood vessel and the consequences of interactions of angiogenesis regulators with vessel cells. Alternatively, primary endothelial and smooth muscle cells grown in culture, which have been applied extensively to the study of angiogenesis (Kader et al., 2000), do not reflect the regular organisation of a vessel wall (Vernon et al., 1995).

Angiogenesis can also be studied ex-vivo by culturing explants of aorta 'rings' in biomatrix gels. Thin sections of rat aorta embedded in either collagen or fibrin matrices, in the absence of exogenous growth factors are capable of forming new blood vessels that sprout from the vessel wall (Diglio et al., 1989; Nicosia and Ottinetti, 1990). The injury to the aorta and the release of local growth factors appear to induce a cascade of responses leading within a few days to the migration and proliferation of endothelial cells and fibroblasts which assemble into thin elongated vascular sprouts. Subsequently, smooth muscle cells appear to differentiate into pericytes and migrate from the aorta along the sprouts to initiate the formation of the vascular media. Within 10 days, the angiogenic process is completed and the sprouts are ready to form anastomosic connections when directed towards a neighbouring vessel (Nicosia and Villaschi, 1999, 1995). Exogenous growth factors are not required for the entire process, and vascular endothelial growth factor (VEGF), which is produced transiently by the aorta ring, appears to be involved in the sprouting cascade (Nicosia et al., 1997). Other, yet unidentified, factors produced endogenously by the injured aorta may also participate in angiogenesis. The aorta ring explant model has been applied extensively for investigations of pro- and anti-angiogenic factors with potential therapeutic applications. Polypeptides, such as angiostatin, thrombospondin, and transforming growth factor-β (TGF-β), have been shown to serve as inhibitors, while growth factors, such as VEGF and fibroblast growth factor (FGF), stimulated the angiogenic process (Nicosia and Villaschi, 1999; Iruela-Arispe and Dvorak, 1997; Stevenson et al., 1998; Kruger et al., 2000). The aorta ring model provides a heterotypic yet simple system for studying the genes that control the angiogenic process. The aim of the present study was to develop the rat aorta explant model for the purposes of viral mediated gene transfer, in order to study the tropism of three viral vectors to the cells constituting the blood vessels. Optimal conditions were first defined for infection with adenovirus, vaccinia virus and retrovirus vectors, which express the β-gal reporter gene, and subsequently, the modified gene transfer model was validated by studying the activities of VEGF and IFNβ genes transduced by viral vectors.

2. Materials and methods

2.1. Rat aorta ring explant cultures

Rat (Spraque–Dawley, 7 weeks old) aorta ring explant cultures were prepared by modification of the standard method used by Nicosia and Ottinetti (1990). About 1 mm-long aortic rings were sectioned and rinsed thoroughly in four consecutive washes of Phosphate Buffered Saline (PBS), followed by plating in 24-well plates coated with 0.4 ml rat tail type-I collagen matrix solidified at 37 °C (15 min). Aorta rings, rinsed with liquid-phase collagen, were then stacked on the collagen layer for 10 min at 37 °C and another upper collagen layer (0.2 ml) was added and solidified for 10 min in the incubator. After collagen gellation, 0.3 ml endothelial serum free medium (SFM) (Gibco BRL, NY, USA) was added to each well. About 1–2 days later, cells started to sprout from

the explants, forming microvessel-like structures within 7 days of culture.

2.2. Viral vectors

The M-MuLV retrovirus encoding the β-gal gene (MuLV-β-gal) and the M-MuLV encoding the IFNβ gene (MuLV-mIFNβ) were constructed using the LXSN vector (Miller and Rosman, 1989) with the β-galactosidase gene or mouse IFNβ cDNA, respectively, under control of the 5′ LTR promoter. The MuLV-based retroviral vector (LXSN) containing human $VEGF_{165}$ (Neufeld et al., 1999), designated MuLV-VEGF, was a gift from Dr M.Y. Flugelman (Lady Davis-Carmel Medical Center and the Bruce Rappaport School of Medicine, The Technion, Haifa, Israel).

The transfer vectors were packaged by co-transfection (calcium phosphate) with the pCL-Ampho packaging construct in 293 cells. Supernatants containing the viruses were collected after 2 days, and the virus titer ($\sim 5 \times 10^5$ infectious units/ml) was determined after infection to NIH-3T3 cells and selection with G418 (Naviaux et al., 1996).

The adenoviral vector (Ad5CMVlacZ) containing the reporter gene, β-gal, under control of the CMV promoter was defective at the E1A gene and was propagated in 293 cells (Graham and Prevec, 1991). Virus titer (5×10^5 pfu/ml) was determined on HeLa cells. The vaccinia expression vector, encoding the β-gal gene under control of the vaccinia promoter, vSC9 (Chakrabarti et al., 1985), was a gift from Dr B. Moss (NIH, Bethesda, MD). The virus titer (5×10^5 pfu/ml) was determined on HeLa cells.

2.3. X-gal staining of tissue explants

Explants in collagen were fixed for 20 min in 0.2% glutaraldehyde, 2% formaldehyde, 2 mM $MgCl_2$, 0.05 M $NaPO_4$ buffer (pH 7.4), washed three times (15 min each) in 0.05 M $NaPO_4$ buffer (pH 7.4), 2 mM $MgCl_2$, 0.02% NP40, and incubated (from 2 h to overnight) at 37 °C in 0.05 M $NaPO_4$ buffer (pH 7.4), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM $MgCl_2$, and 20 mg/ml of X-gal substrate (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside).

2.4. ONPG assay

The *O*-nitrophenyl β-D-galactopyranoside (ONPG) substrate (Larkin et al., 1996; Ballard et al., 1995) was used to quantitate β-galactosidase enzyme activity in the transduced tissues. Explants were minced and lysed by three freeze–thaw cycles and 2 min of sonication. Total protein content of the explant lysates was determined by the Bradford method (Bio-Rad). Tissue extracts (20 μg) were incubated with vol./vol. of the enzyme substrate [ONPG (1.33 mg/ml, Sigma, MO, USA) in 1 ml of 200 mM sodium phosphate (pH 7.4), 2 mM $MgCl_2$, 100 mM β-mercaptoethanol]. Absorbance (405 nm) was measured by an ELISA plate reader. Reactions were run in duplicates, and enzyme-specific activities were determined from the linear part of time kinetic curves.

2.5. Cell viability (MTT) assay

To assess the viability and proliferation of cells in the culture, the MTT assay was performed essentially as described previously (Miller and McDevitt, 1991). The assay is based upon the ability of mitochondria to convert MTT (3-[4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide]) (Sigma, MO, USA) into a blue formazan product. The optical density at 492 nm was measured in an ELISA plate reader (Organon Teknika, The Netherlands) with a reference wavelength of 650 nm.

2.6. Quantitation of angiogenesis

Sprout formation from the aorta rings was quantitated by counting the number of neovessels according to published criteria (Nicosia and Ottinetti, 1990). Branched vessels and loops were counted as two sprouts. Neovessels were counted blindly by two different investigators to confirm the results. Standard errors (S.E.) reflect differences in counts of sprouts in five replicate determinations.

2.7. RNA extraction and RT-PCR analysis

Total RNA was extracted by TRI-reagent (Sigma, MO, USA). Minus-strand cDNA was prepared using oligo(dT)15 with M-MLV reverse transcriptase (Gibco-BRL, NY, USA), as described previously (Ashhab et al., 2001). The expression of the human $VEGF_{165}$ was detected using the following gene-specific primers: Fr: 5′-AATGACGAGGGCCTGGAGTG and Rv: 5′-CAAATGCTTTCTCCGCTCTGAG. Both primers were selected such that they do not interreact with the rat endogenous VEGF gene sequences. PCR (30 cycles) was carried out by denaturation at 94 °C for 30 s, annealing at 55.5 °C for 45 s and extension at 72 °C for 60 s. The DNA products were resolved by electrophoresis on agarose gel (1.5%).

2.8. Vesicular stomatitis virus plaque reduction assay

To assess the expression of IFNβ in the transduced cultures, the vesicular stomatitis virus (VSV) plaque-forming–reduction-assay was carried out essentially as previously described (Stewart et al., 1972). The assay is based on the potency of IFN to render cell cultures resistant to the cytopathic effect of viral infection. About 8 days post transduction with MuLV-mIFNβ, the cultures were infected with VSV (7.5×10^4 pfu/ml), and cell viability was assayed by MTT 2 days post-infection.

3. Results

The initial attempts to apply the aorta explant model to the transfer of genes, which might have affected the angiogenic process, had failed. In order to test whether the collagen matrix interferes with gene transfer mediated by viral vectors, endothelial cells derived from the rat aorta were grown under a collagen layer and then transduced with retro-or adeno-viral vectors containing the β-gal gene. These experiments showed that the upper layer of collagen acts as a barrier which blocks transduction of the cultured cells by the viral vectors. Consequently, the model was modified as follows.

3.1. First modification

Rat aorta rings were placed on top of a layer of collagen upon which they successfully induced capillary-like sprouts without the need of an upper layer of collagen. To circumvent the deep penetration of sprouts, the collagen layer was made as thin as possible, enabling the virus to infect the shallow embedded sprouts. Infection with either adeno-β-gal vector (Fig. 1A and B) or a retroviral vector, MuLV-β-gal, (Fig. 1C and D) resulted in efficient gene transfer to both the single endothelial cells as well as to the arranged vascular sprouts.

3.2. Second modification

Rat aorta rings were cultured in a liquid medium for up to 13 days before transduction and subsequent embedding in collagen gels. Each aorta ring was housed in a well of 96-well plates coated with a 1% soft agar layer containing 0.15 ml SFM. The wells were coated with agarose to avoid attachment of the ring and corresponding cells. The purpose of the preincubation in a liquid medium was to investigate the possibility of gene transfer to the endothelial cell mono-layer within the lumen of the aorta prior to neo-vessel sprouting. As shown in Fig. 1E, MuLV-β-gal efficiently infected the endothelium within the lumen, after 5 days of preincubation on agarose, in the absence of any apparent sprouting.

In order to select the optimal viral vector for gene transfer, the efficiencies of gene transfer by the different viral vectors were compared. For this purpose, aorta rings were grown on collagen (first modification) and infected at increased multiplicities with MuLV-β-gal, adeno-β-gal or vaccinia-β-gal (Fig. 2). The explant cultures were harvested, cell extracts prepared, and β-gal enzyme activity was quantitated with the ONPG substrate. Increased multiplicities of infection of the three viral vectors resulted in a near linear elevation in β-gal enzyme-specific activity. At a high viral multiplicity, the extent of β-gal gene expression reached a

plateau, with the adeno vector showing the highest level of gene transfer (Fig. 2).

However, while aorta explant infection with the adenoviral vector was very efficient, it appeared to result in endothelial cell death and, subsequently, in the inhibition of the angiogenic process, as indicated by rounding of the X-gal-stained cells, which formed the newly sprouting vessels (Fig. 1B). This result corresponds to our earlier observations related to cell toxicity after adenoviral vector infection of other tissues. The vaccinia vector had a similar effect on the morphology of infected cells (data not shown). Nevertheless, retroviral vector, at similar multiplicity of infection used for adenovirus and vaccinia vectors ($\sim 10^5$ infectious units), did not appear to induce any morphological cell damage (Fig. 1D). Due to the cytopathic effect of adenovirus and vaccinia vectors, all subsequent studies of gene transfer in the aorta ring model were carried out with the retroviral vector.

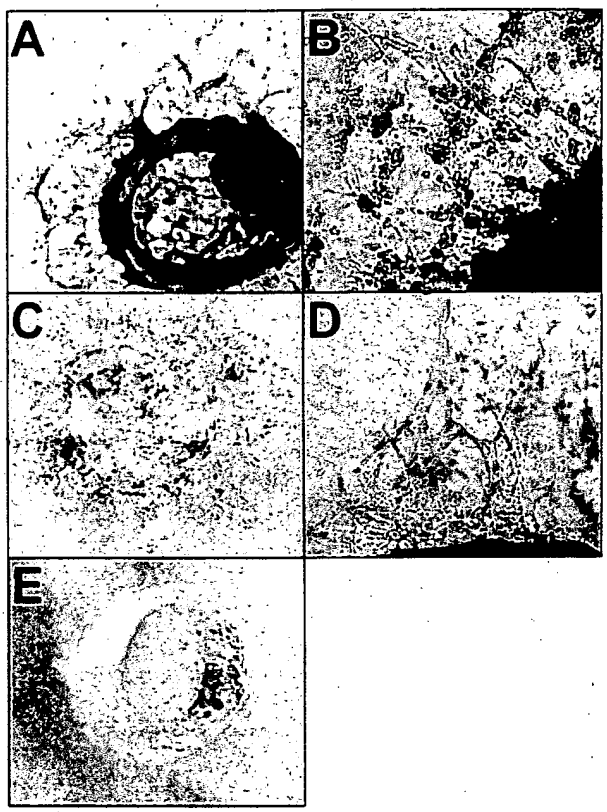

Fig. 1. Viral-mediated β-gal gene transfer to aorta rings. First modification, rat aorta rings grown on a thin layer of collagen (0.2 ml in 16 mm wells) were infected for 2 h with adeno-β-gal (A, B), or MuLV-β-gal (C, D) vectors (10^5 infectious units per ring) and stained after 24–48 h with X-Gal (A and C, stereoscope). After 24 h of infection, the adeno-β-gal induced rounding of X-Gal stained blue cells, the typical phenotype of cell death (B, × 100 magnification). MuLV-β-gal infection (48 h) did not affect cell morphology (D, × 100 magnification). Second modification: after 5 days of pre-incubation on agarose layer (37 °C, 10% CO_2), several aorta rings were infected together with MuLV-β-gal-containing medium (1.5 ml, 5×10^5 cfu/ml) for 2 h, rinsed with SFM, and returned to agarose-coated plates for another 24–48 h. This resulted in β-gal expression in aorta lumen endothelial cells (E, stereoscope).

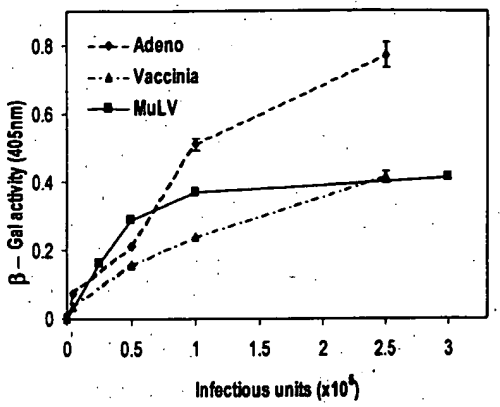

Fig. 2. Efficiency of infection of aorta rings with adenoviral, vaccinia, and retroviral vectors. β-Gal enzyme activity was quantitated with the ONPG substrate following infection at different viral multiplicities per ring. Protein content of the extracts was determined by the Bradford method, and amounts of the ONPG product were corrected per 20 μg protein. Aorta explants grown on collagen layer (first modification) were infected with MuLV-β-gal ($0.1-3 \times 10^5$ cfu) at day 3 (■)and harvested at day 6 post seeding. Explants were infected at day 5 with vaccinia-β-gal ($0.1-2.5 \times 10^5$ pfu) (▲) and adenoviral-β-gal (◆) and harvested at day 6 post seeding. Error bars represent the standard error (S.E.) of the mean value of five replicate determinations.

In order to determine the optimal conditions for retroviral-mediated gene transfer and to assess the changes that had occurred during the angiogenic process that might affect transgene expression, the aortic rings were infected on a collagen layer (first modification) at various times post seeding for a constant period of 3 days. Enzyme, β-Gal, activity was assayed on extracts of the infected explants (Fig. 3A, 3 days P.I.). The results indicated that the optimal time of transduc-

tion is 3–6 days post seeding, when the neo-vessels are already visible in the collagen matrix. To test how progression of the angiogenic process affects prolonged transgene expression, the aortic rings were infected at different intervals post seeding, and all were harvested on the same day, i.e. 13 days after seeding. The results (Fig. 3A, 13 days P.S.) suggested that transgene expression is maintained at a steady state for at least 12 days (the duration of the experiment). Since retroviral-mediated gene transfer requires active cell division, we also tested the kinetics of DNA synthesis in the aorta ring cultured in liquid medium (Fig. 3B). While low DNA synthesis (BRDU incorporation) was detected at day 1, extensive DNA synthesis was recorded between 3 and 13 days post seeding in liquid medium. The two observations; infection of endothelial cells in the lumen (Fig. 1E) and DNA synthesis (Fig. 3B), indicate that endothelial cells actively proliferate within the aorta lumen prior to their migration out of the lumen and subsequent sprouting.

To test the utility of this gene transfer model, the gene encoding for $VEGF_{165}$ was overexpressed. Aorta rings were infected in liquid medium (second modification) with the MuLV-VEGF vector and after 3 days tested for angiogenic parameters. Microscopic evaluation of the aorta rings after infection with MuLV-VEGF clearly showed enhanced sprouting compared with the control mock-infected aorta (Fig. 4A and B). After transduction with the VEGF gene, quantitation of the new-vessels by counting sprouts under the microscope indicated that there had been a three-fold enhancement of the angiogenic process (Fig. 4C). Interestingly, overexpression of VEGF enhanced sprout formation to even higher extent (15.4-fold), when the aortic rings were seeded in a matrix composed of a mixture of collagen/agarose (Fig. 4C).

Since the viral preparation, the conditioned medium obtained after packaging the MuLV-VEGF vector in the 293 cell line, may contain recombinant VEGF protein and other soluble growth factors, a control experiment was conducted to test whether treatment of the aorta ring with UV-inactivated MuLV-VEGF enhanced the angiogenic process. Fig. 4D shows that the UV-inactivated MuLV-VEGF preparation had lost its pro-angiogenic capacity, indicating that transduction of the VEGF gene is, indeed, responsible for the angiogenesis illustrated in Fig. 4A–C.

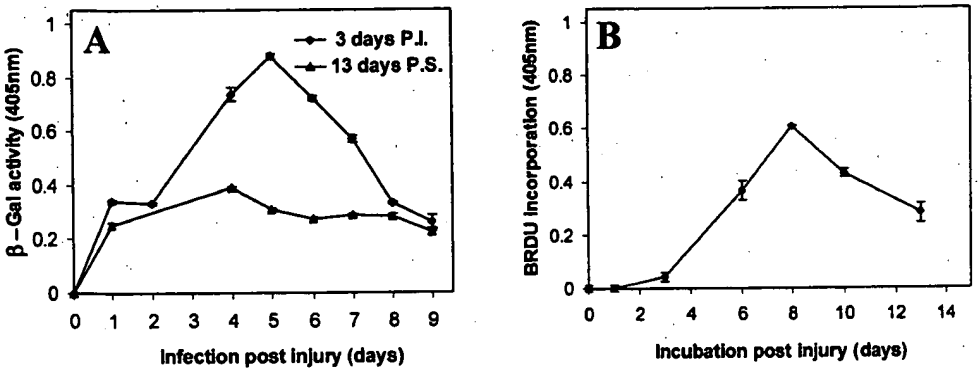

Fig. 3. Optimisation of MuLV vector mediated gene transfer to the aorta rings. (A) Optimisation of MuLV gene transduction during angiogenesis: aorta rings grown on collagen layer (first modification) were transduced with the MuLV-β-gal vector (0.5 ml, 10^5 cfu/ml) using two schedules. In the first schedule, a constant period of time (3 days) was assigned for the infection; i.e. cultures were infected at different intervals post seeding and harvested after an additional 3 days (3 days P.I.). In the second schedule, explants were infected at different days after seeding on collagen and all cultures were harvested at day 13 post seeding (13 days P.S.). β-Gal expression was assayed by ONPG and corrected per 20 μg protein extract. (B) Kinetics of DNA synthesis in the aorta ring cultured in liquid medium: Aorta rings that had been preincubated up to 13 days on soft-agar coated plates (second modification) were transferred to new plates and treated overnight with 5-bromo-2′-deoxyuridine (BRDU, Boehringer, Mannheim, Germany). BRDU test was performed according to the manufacturer's instructions. Error bars represent the S.E. of the mean value of five replicate determinations.

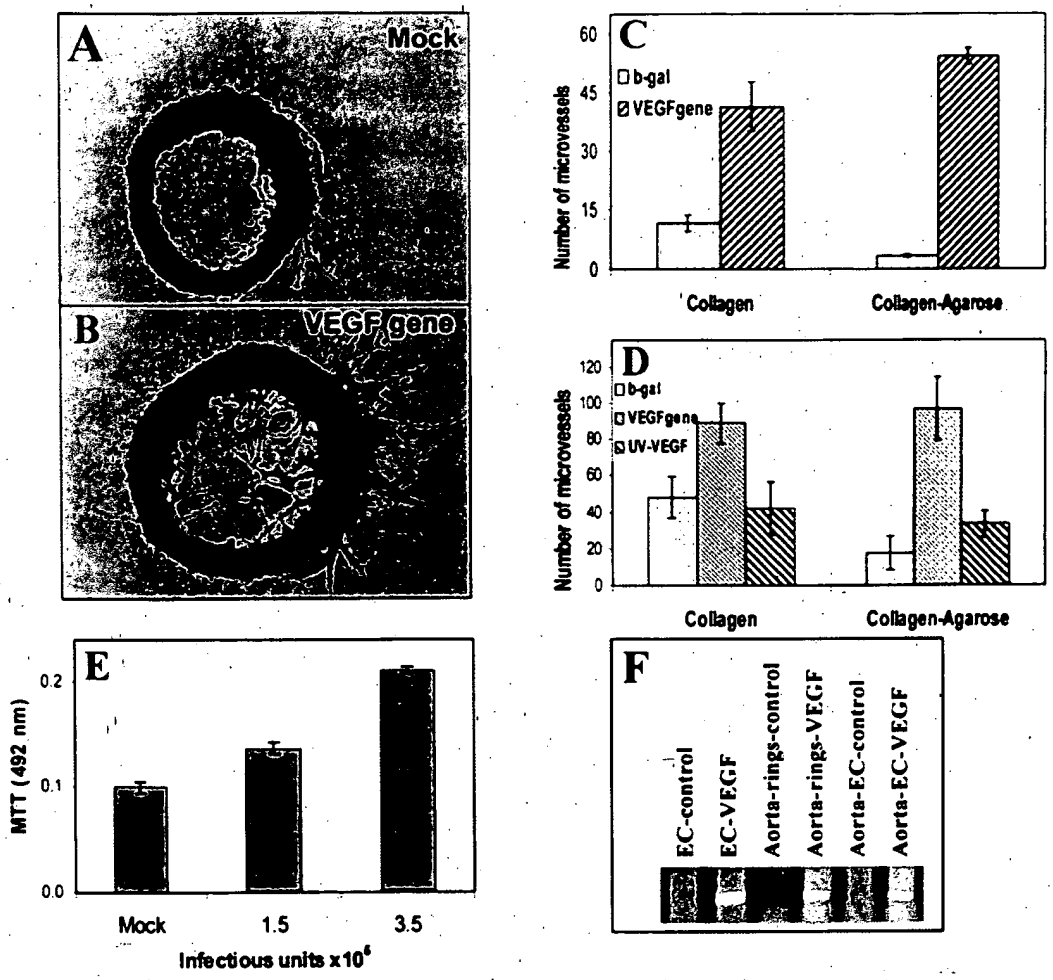

Fig. 4. VEGF gene expression enhances endothelial cell proliferation and aorta sprout formation. (A–C) Aorta rings were pre-incubated for five days on agarose (second modification) and then transduced with MuLV-VEGF (7.5×10^5 cfu/15 aorta rings in 3 cm plate). Immediately after infection, the transduced aorta rings were embedded either in collagen or in a mixture (1:1, v/v) of collagen and sea-plaque agarose (1%) mixed prior to gellation at 37 °C. Three days after transduction, microvessel formation was photographed with a stereoscope (A and B, aorta grown in collagen) and quantitated by direct counting the number of neovessels (C). (D) In order to validate that the pro-angiogenic activity of the MuLV-VEGF preparation is due to overexpression of the gene and not to VEGF protein in the virus preparation, aorta were infected (second modification) with UV-inactivated MuLV-VEGF preparation. For UV-inactivation, MuLV viral vectors containing β-gal or VEGF genes (5 ml in 10-cm culture plates) were irradiated for 45 min by an ultraviolet (UV) transilluminator (Ultra Violet Products, INC., San Gabriel, California, USA) at 260 nm from a distance of 20 cm, and viral inactivation was validated by titrating MuLV-β-gal on NIH-3T3 culture cells. (E) Aorta rings grown on collagen layer (first modification) were transduced (at day 4 post seeding) with MuLV-VEGF (1.5×10^5 and 3.5×10^5 cfu per aorta ring). Five days after infection, total cell proliferation in the plate was assessed by the MTT assay. Mock controls were achieved by using the MuLV-β-gal vector. Error bars represent the S.E. of the mean value of five replicate determinations. (F) RT-PCR analysis performed on mRNA extracted from VEGF transduced endothelial cells (lanes 1–2), VEGF transduced aorta rings (lanes 3–4) and endothelial cells derived from VEGF transduced aorta rings (lane 5–6). Lanes 1, 3, 5 are controls (transduced with β-gal) and lanes 2, 4, 6 were transduced with MuLV-VEGF.

The proliferative activity of the VEGF transgene could also be noticed when all the viable cells in the explant culture, whether in sprout or as single cells, were quantitated by the MTT colorimetric assay (Fig. 4E). This observation further indicates that the endogenous rat VEGF produced in the aorta organ culture is suboptimal for cell proliferation and sprouting, and that transgene VEGF overexpression determines levels of cell proliferation in a dose-dependent manner. To validate further that indeed the transgene product, VEGF, is responsible for endothelial cell proliferation, three lines of independent experiments were carried out, first, primary endothelial cells derived from rat aorta were transduced in culture by the same MuLV-VEGF vector. Stable cell clones were selected for G418 resistance and used for proliferation test using the MTT assay. The results indicate threefolds higher proliferation of the MuLV-VEGF transduced cells as compared with control cells transduced with a reporter gene, β-gal (data not shown). Second, the MuLV-VEGF transduced endothelial cells produced capillary structures when seeded on a matrigel matrix (data not shown). Third, mRNA product of the $VEGF_{165}$ trans-gene was detected, by RT-PCR, in the transduced aorta rings but not in the control rings (Fig. 4F). Furthermore, mRNA expression of the $VEGF_{165}$ trans-gene was detected, 2 weeks post infection, in endothelial cell cultures derived from transduced aorta rings (Fig. 4F).

In order to validate further the applicability of the ex-vivo aorta model for gene transfer, we exploited the capacity of IFN to render cells resistant to the consequence of cytopathic viral infection. To establish the species-specificity of rat cells to the available IFN genes, we first compared human and mouse IFN beta genes for the protection of rat endothelial cells against the cytopathic effect of VSV. Cultured rat endothelial cells were transduced with the MuLV vector containing either the mouse or human IFNβ genes and infected with VSV. The results showed that mouse IFNβ protected rat endothelial cells by 55%, while human IFN was not effective against the VSV cytopathic effect. Subsequently, aorta rings in a collagen matrix (first modification) were transduced with the mouse IFNβ gene (MuLV-mIFNβ) and cultures were infected with VSV eight days later (Fig. 5). The results demonstrated significant protection against the VSV cytopathic effect for the aorta cells expressing the IFNβ transgene, when compared with control β-gal gene-expressing aorta infected with VSV.

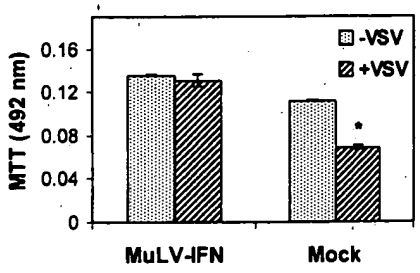

Fig. 5. IFNβ gene transduction renders aorta cultures resistant to VSV cytopathic effect. Aorta rings grown on collagen layer (first modification) were transduced (at day 3 post seeding) with MuLV-mIFNβ (3.5×10^5 cfu per aorta ring). Eight days after transduction, cultures were infected with VSV (7.5×10^5 pfu per aorta ring). Two days later, cell viability was assessed by the MTT assay. Mock control was achieved by using MuLV-β-gal vector. *, $P < 0.001$ as compared with control and as compared with MuLV-mIFNβ-aorta infected with VSV (ANOVA). Error bars represent the S.E. of the mean value of five replicate determinations.

4. Discussion

There are three major experimental approaches for the study of angiogenesis, cultured endothelial cells (Benelli and Albini, 1999); ex vivo organ explant culture (Nicosia and Ottinetti, 1990); and animal models (Ribatti and Vacca, 1999; Nabel and Nabel, 1994; Nabel et al., 1993), each representing different aspects of this complex biological process. The ex-vivo system of a three-dimensional blood vessel explant has provided a relatively simple heterotypic model of angiogenesis, involving all cells and the extracellular components constituting the vessel wall (Nicosia and Ottinetti, 1990).

In this study the ex vivo aorta ring model was modified in order to allow efficient viral mediated gene transfer to the sprouting neo-vessels. Two major modifications were introduced to the standard procedure, (i) placing the aorta ring on top

of a thin layer of collagen, such that the angiogenic tissue would be accessible to the viral vector; and (ii) infection of the aorta rings prior to embedding into the collagen matrix. Under these conditions, the aorta and the vessel sprouts were accessible to viral infection and gene transfer, either during the process of sprouting (first modification) or before the initiation of the sprouting cascade (second modification).

Initially, three viral vectors were tested as potential vehicles for gene transfer, adenoviral; vaccinia and retroviral vectors. The two DNA viruses, while being efficient in gene transfer, both to the lumen of the aorta and to the neo-vessels, appear to cause morphological changes in the infected cells. The adenoviral vector used in this study is a first-generation viral vector that was deleted from the E1A gene and, therefore, it is unable to complete a replication cycle in the endothelial cells (Graham and Prevec, 1991). Nevertheless, since some viral genes were expressed, it appears to have induced cellular damage in the infected endothelium. The vaccinia vector is a replication competent virus and, therefore, cytopathic to the endothelium. Application of adenoviral vector for gene transfer to the vasculature in animal models has been extensively investigated. In-vivo infection and gene transfer are efficient with this vector, but is usually associated with an inflammatory response by the host and with cellular damage (Crystal, 1995). Although the retroviral vector, MuLV, infected the neo-vessels somewhat less efficiently, compared with the two DNA viruses, it did not appear to interfere with the angiogenic process, even at high multiplicities of infection (Fig. 1D).

Since retroviral infection requires cell proliferation, transduction of the reporter β-gal gene can serve as a sensitive tool to specifically mark the subpopulation of dividing endothelial cells during the angiogenic process. Using this parameter, our experiments indicated that some endothelial cell proliferation occurs as early as 1 day post injury. This assay appears to be even more sensitive than labelling of cell DNA with BRDU; the latter assay did not detect cell proliferation at 1 day post seeding. Furthermore, marking of the proliferating cells at different intervals of the angiogenic cascade may reveal stages of differentiation of the different cells participating in this complex process. Our data suggest that endothelial cell proliferation within the aorta lumen precedes cell migration out of the lumen into the surrounding matrix, as shown by β-gal expression in the luminal endothelial cells (Fig. 1E). The ability to mark genetically those early proliferating endothelial cells with a reporter gene paves the way to monitoring the fate of early dividing cells during angiogenesis. The subpopulation of genetically marked cells could be isolated now by cell sorting (FACS), and their differentiation stage can be determined.

To validate the applicability of the ex vivo model for functional gene transfer, the pro-angiogenic capacity of VEGF was exploited. VEGF is a major regulator of the angiogenic process; stimulating endothelial cell proliferation, cell migration, sprouting and vessel permeability (Neufeld et al., 1999). It is considered an important growth factor in the aorta ring model, as it is produced transiently during the angiogenic process (Nicosia and Villaschi, 1995). The enhancement of sprout formation after VEGF over-expression confirms successful gene delivery and expression in the aorta. Furthermore, it also suggests that production of endogenous VEGF is a limiting factor in the angiogenic process, at least in the ex vivo model.

The finding that mIFNβ over-expression renders the aorta explant cultures resistant to VSV infection confirms further the applicability and utility of the ex-vivo model for functional gene transfer. Likewise, this model can serve to investigate endothelium protection against blood-borne pathogenic viruses, which infect endothelial cells during spreading in-vivo (Friedman, 1989). Protection of the endothelium by IFN over-expression may, therefore, be considered as a therapeutic approach against certain infections.

In summary, the ex-vivo aorta culture is now adaptable to viral mediated gene transfer for the study of genes that may regulate and modify the complex angiogenic process. This model should be especially amenable to the study of new anti-angiogenic factors, as we find no background of inhibitory activities during the cultivation. In contrast, the endogenous rat VEGF produced might

over-shadow weak pro-angiogenic activities. Thus, this system opens new avenues for the development of gene therapy approaches for preventing neointimal growth, restenosis, of the blood vessel.

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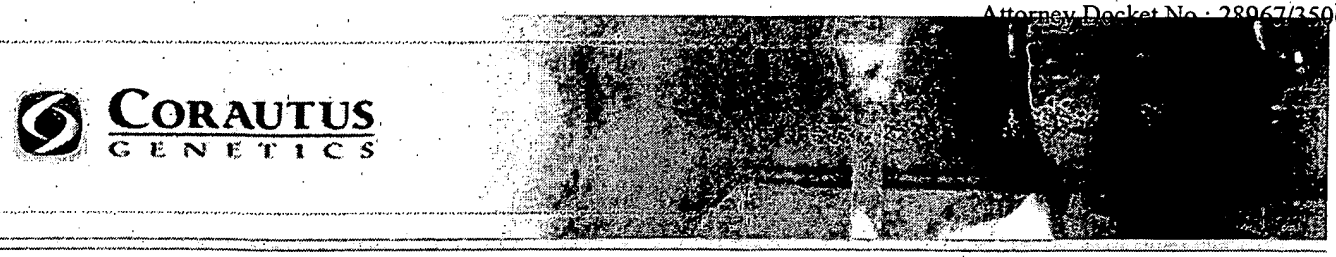

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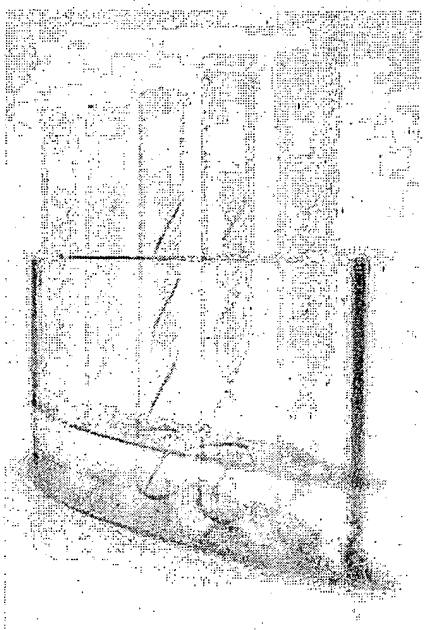
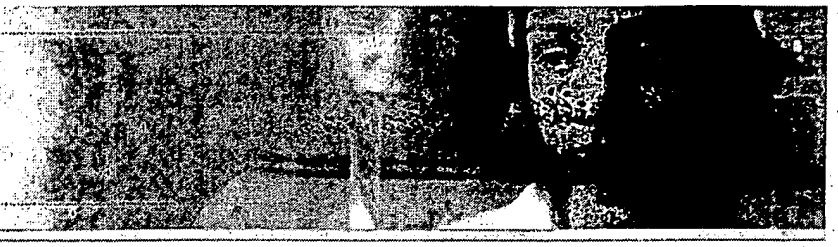


Therapeutic Development Program

Therapeutic Angiogenesis and VEGF-2

Corautus Genetics, Inc. utilizes a cath eter-based administration of angiogenic growth factors to promote the development of supplemental collateral blood vessels, a process known as therapeutic angiogenesis. Several agents have demonstrated the ability to induce angiogenic activity. Both protein and gene stimulate anglogenesis in animal models of ischemia. Corautus Genetics uses VEGF-2 which is delivered via naked DNA plasmid, a non-viral vector. VEGF-2 appears to be selective for endothelial cells which means its activity and "stimulation" appears to be limited to the recruitment of endothelial cells within the area surrounding the injection site. The recruited endothelial cells begin to express protein which in turn stimulates the growth of new blood vessels by promoting the migration and proliferation of endothelial cells.

CORAUTUS GENETICS



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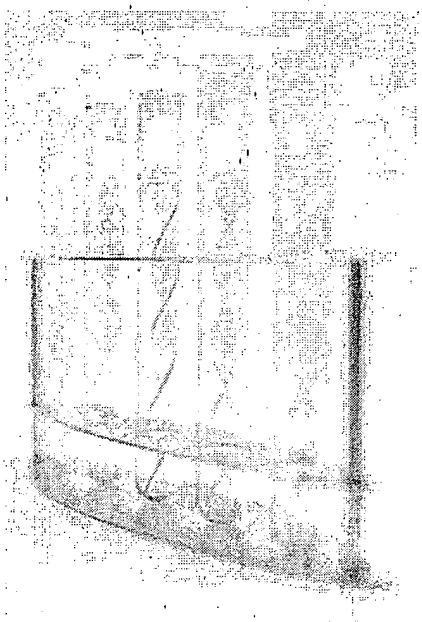

Therapeutic Development Program

Phase IIb Clinical Trial

The Phase IIb clinical trial is designed to treat Class III and IV angina patients who are not candidates for current revascularization techniques and for whom surgery is not advisable.

The 404 patients are randomized to placebo or one of three active treatment groups. The Phase IIt clinical trial will provide information regarding long-term safety and benefits of treatment in patient with Class III and IV angina. And, it will provide information to determine the optimum dosage of VEGF-2.

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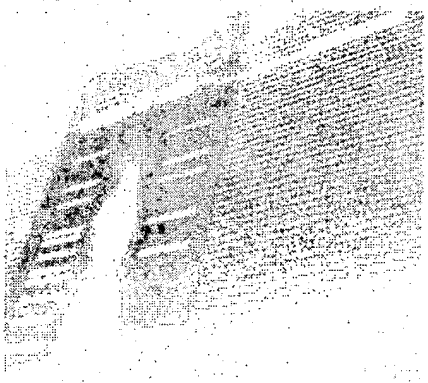

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News Release

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Corautus Genetics Signs Manufacturing Agreement with Qiagen and Strathmann Biotec AG for VEGF-2 Plasmid Production

ATLANTA--(BUSINESS WIRE)--Jan. 8, 2004--Corautus Genetics Inc. (AMEX: CAQ -) has signed a manufacturing agreement with Qiagen NV (Nasdaq : QGENF; Frankfurt, Prime Standard: QIA) and Strathmann Biotech AG to produce VEGF-2 plasmid for anticipated Phase III trials and future commercial use. The Company has in inventory the VEGF-2 plasmid required for its upcoming Phase IIb clinical trial.

Richard Otto, Chief Executive Officer of Corautus, commented, "Signing this manufacturing agreement is an important building block as we look beyond our Phase IIb trial to plan for Phase II trials and projected future commercialization of our product. We have decided to enter into this agreement now because it gives us the assurance that if the timelines for the trials and commercialization get accelerated, we are guaranteed a slot in the manufacturing cycle. It also enables us to do advanced planning for quality control and assurance that will be part of this process."

Mr. Otto continued, "We thoroughly evaluated various potential manufacturing partners, and are confident that QIAGEN and Strathmann are best-suited to meet Corautus' scientific specifications, quality requirements and development schedule."

The VEGF-2 plasmid will be produced at Strathmann's newly-built facility in Dengelsberg, Germany The facility was designed and built specifically for plasmid DNA production.

Bert Behnke, Chief Scientific Officer of Strathmann, stated, "We are very pleased that Corautus ha: chosen us to manufacture their product which will be used to help treat cardiovascular disease. Ou state-of-the-art facilities are ideal for producing the high-quality VEGF-2 plasmid that will be required to comply with the standards of the regulatory agencies."

Corautus' therapy, which is considered regenerative medicine, seeks to treat the underlying causes of severe coronary artery disease through direct injection of VEGF-2 into ischemic cardiac muscle. Based on data from earlier clinical trials - Phase I and IIa have been completed - Corautus believes that when VEGF-2 is introduced into ischemic cardiac muscle, it will stimulate the growth of new blood vessels and improve cardiac function. The VEGF-2 is delivered via a non-viral vector system in which the naked DNA plasmid is injected directly into the tissue where the gene transfer occurs. The Company believes that there are several advantages to this over other gene transfer delivery systems including minimal side effects and the fact that the gene is not inserted into the genome.

Dr. Joachim Schorr, QIAGEN's Managing Director and Senior Vice President Global Research & Development, noted, "We have completed numerous projects with Strathmann for pre-clinical and clinical trials. Strathmann's strong plasmid DNA manufacturing capabilities combined with QIAGEN' expertise in nucleic acid purification, reflected in our products and technologies, addresses the highest requirements in purity and efficiency of plasmid DNA manufacturing for gene therapy' applications."

About Corautus Genetics

Corautus Genetics Inc. is a clinical stage biopharmaceutical company dedicated to the developmen of gene transfer therapy products for the treatment of severe cardiovascular and peripheral vascular disease. Corautus is currently developing and testing a gene transfer product using the Vascular Endothelial Growth Factor 2 (VEGF-2) gene to promote therapeutic angiogenesis in ischemic muscle. In July 2003, Corautus entered into a series of agreements with Boston Scientific Corporation to fund, develop, commercialize and distribute the VEGF-2 gene therapy products.

About QIAGEN

QIAGEN N.V., a Netherlands holding company with subsidiaries in Germany, the United States, Japan, the United Kingdom, Switzerland, France, Italy, Australia, Norway, Austria and Canada, ' believes it is the world's leading provider of innovative enabling technologies and products for the separation, purification and handling of nucleic acids. The Company has developed a comprehensive portfolio of more than 320 proprietary, consumable products for nucleic acid separation, purification and handling, nucleic acid amplification, as well as automated instrumentation, synthetic nucleic acid products and related services. QIAGEN's products are sold i more than 42 countries throughout the world to academic research markets and to leading pharmaceutical and biotechnology companies. In addition, the Company is positioning its products for sale into developing commercial markets, including DNA sequencing and genomics, nucleic acid based molecular diagnostics, and genetic vaccination and gene therapy. QIAGEN employs approximately 1,600 people worldwide. Further information on QIAGEN can be found at www.qiagen.com.

About Strathmann Biotech AG

Strathmann Biotec AG is an expanding biotechnology company based in Hamburg, Germany. Founded in 1987 as Pharma Biotechnologie Hannover, it was one of the first manufacturers of recombinant proteins in Germany. Since 1997 it is part of the Strathmann Group and a subsidiary of Strathmann AG, a leading mid-sized pharmaceutical company in Germany. Strathmann Biotec offers its contract development and contract manufacturing services to international customers from the pharmaceutical and biotechnology industry. The company has long-standing experience ir the area of protein expression systems, plasmid DNA technologies, downstream processing as well as manufacturing. Further information on Strathmann Biotech AG can be found at http://www.strathmann-biotec-ag.de/en/company/comphome.shtml.

Forward Looking Statement

This press release may contain forward-looking statements made pursuant to the safe harbor provisions of the Private Securities Litigation Reform Act of 1995. Such statements are subject to certain factors, risks and uncertainties that may cause actual results, events and performances to differ materially from those referred to in such statements. These risks include statements which address operating performance, events or developments that we expect or anticipate will occur in the future, such as projections about our future results of operations or our financial condition,

benefits from the alliance with Boston Scientific, synergies from the merger between GenStar and Vascular Genetics, research, development and commercialization of our product candidates, anticipated trends in our business, approval of our product candidates and other risks that could cause actual results to differ materially. These risks are discussed in Corautus Genetics Inc.'s Securities and Exchange Commission filings, including, but not limited to, the risks discussed in Corautus' Annual Report on Form 10-K (File No. 001-15833) filed March 28, 2003, which are incorporated by reference into this press release.

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"Safe Harbor" Statement under the Private Securities Litigation Reform Act of 1995: Statements in this press release regarding CORAUTUS GENETICS INC's business which are not historical facts are "forward-looking statements" that involve risks and uncertainties. For a discussion of such risks and uncertainties, which could cause actual results to differ from those contained in the forward-looking statements, see "Risk Factors" in the Company's Annual Report or Form 10-K for the most recently ended fiscal year.

One-Year Follow-Up of Direct Myocardial Gene Transfer of Vascular Endothelial Growth Factor-2 Using Naked Plasmid Deoxyribonucleic Acid by Way of Thoracotomy in No-Option Patients*

F. David Fortuin, MD, Peter Vale, MD, Douglas W. Losordo, MD, James Symes, MD, Giacomo A. DeLaria, MD, J. Jeffrey Tyner, MD, Gary L. Schaer, MD, Robert March, MD, R. Jeffrey Snell, MD, Timothy D. Henry, MD, Joseph Van Camp, MD, John J. Lopez, MD, Wayne Richenbacher, MD, Jeffrey M. Isner, MD†, and Richard A. Schatz, MD

This phase I open label, dose-escalating study shows that gene transfer of vascular endothelial growth factor-2 naked deoxyribonucleic acid by direct myocardial injection by way of thoracotomy in patients with Canadian Cardiovascular Society class 3 or 4 angina is feasible and safe. The procedure is well tolerated, with few major adverse cardiac events at 1 year, and without complications directly related to gene expression. In this prospective, nonblinded study, the procedure is associated with clinical improvement; however, there was no angiographic evidence of angiogenesis and there is a great potential for a sham or placebo effect in the study patients. A randomized phase III trial is underway that will help determine the efficacy of vascular endothelial growth factor-2 gene transfer in "no-option" patients. ©2003 by Excerpta Medica, Inc.

(Am J Cardiol 2003;92:436–439)

A phase I clinical study has shown that direct myocardial gene transfer of naked plasmid deoxyribonucleic acid containing the sequence encoding the angiogenic growth factor vascular endothelial growth factor (VEGF)-1 in patients with angina pectoris is safe, decreases angina, and improves myocardial perfusion.[1,2] In vitro and animal studies demonstrate that VEGF-2 has similar, but not identical, properties as VEGF-1.[3–5] The clinical impact of direct myocardial gene transfer of VEGF-2 deoxyribonucleic acid in humans is not known. Thus, we sought to test the long-term safety and clinical effects of direct transthoracic, intramyocardial gene transfer of naked deoxyribonucleic acid encoding VEGF-2 on angina and exercise treadmill test (ETT) times in 30 "no-option" patients with Canadian Cardiovascular Society (CCS) class III or IV angina pectoris refractory to maximal medical therapy and not amenable to conventional revascularization.

TABLE 1 Complications and Serious Adverse Events After Gene Transfer

Complication	0–30 Days	31–365 Days
Death	1 (3%)	0
Myocardial infarction	1 (3%)*	1 (3%)†
Congestive heart failure	2 (7%)*	0
Revascularization	0	1 (3%)†
Stroke	1 (3%)*	0
Arrhythmia	0	0
Proteinuria	0	0
Malignancy	0	0

*†Complications occurred in the same patient.

The Institution Review Board approved the study from each site and written informed consent was obtained from each subject before enrollment. Inclusion criteria were stable CCS class III or IV angina pectoris refractory to maximal medical therapy and not amenable to conventional revascularization, multivessel occlusive coronary artery disease and/or occlusion of ≥1 bypass grafts, and abnormal ETT with concordant stress-induced perfusion abnormalities by single photon emission computed tomography imaging. Exclusion criteria included coronary artery bypass grafting or percutaneous coronary intervention within 3 months before study screening, severe concurrent illness, significant valvular disease or claudication, ejection fraction <25%, history of or clinical evidence of cancer, and the presence of proliferative diabetic retinopathy.

The surgical approach to gene transfer has been previously described.[1] The naked plasmid deoxyribonucleic acid administered (pVGI.1; Vascular Genetics Inc., Atlanta, Georgia) encodes the 52 kDa isoform of human VEGF-2, and patients were treated in a dose-escalating fashion in groups of 10 at total doses of 200, 800, or 2,000 μg. The total volume injected was 8.0 ml for each dose, which was divided and delivered in four 2.0-ml intramyocardial injections.

History, physical exam, and routine blood testing were performed at baseline, 2 weeks, and at 1, 2, 3, 8,

From the Scripps Clinic, Green Hospital, La Jolla, California; St. Elizabeth's Medical Center, Boston, Massachusetts; Rush-Presbyterian-St. Luke's Medical Center, Chicago, Illinois; Hennepin County Medical Center, Minneapolis Heart Institute Foundation, Minneapolis, Minnesota; and the University of Iowa, Iowa City, Iowa. This investigation was supported by Vascular Genetics Inc., Atlanta, Georgia and Cato Research, Ltd, Research Triangle Park, North Carolina. Dr. Schatz's address is: Scripps Clinic, Green Hospital, 10666 North Torrey Pines Road, La Jolla, California 92037. E-mail: RAS1024@aol.com. Manuscript received January 24, 2003; revised manuscript received and accepted May 7, 2003.

*Drs. Isner and Schatz are stockholders of Vascular Genetics, Inc., the sponsor of this trial.

†Dr. Isner died October 31, 2001.

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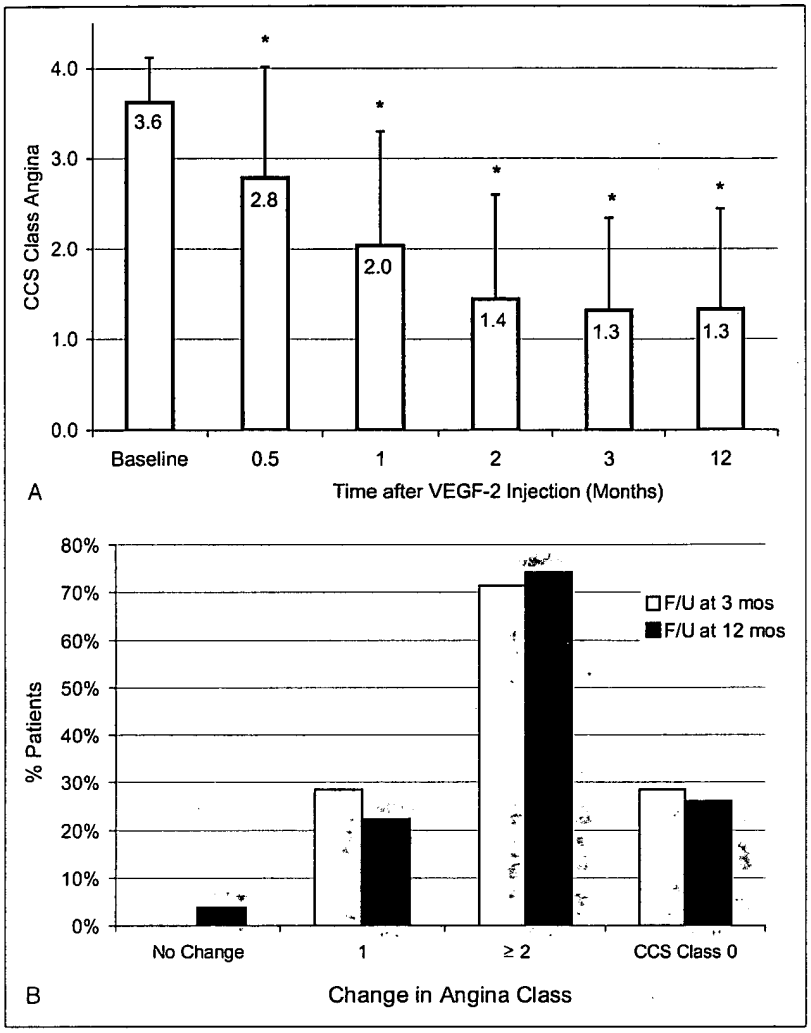

FIGURE 1. *(A)* **Mean CCS angina class. CCS angina decreased significantly at all follow-up visits (n = 29 at baseline; n = 28 at 2 weeks and 3 months; n = 27 at 2 and 12 months). For the 2 patients in whom 12-month data are not available, 1 patient had class 0 angina at 3 months and class 1 at 8 months and the other patient had class 2 at 3 months. *p <0.05.** *(B)* **Relief of Angina. Patients grouped according to magnitude of improvement in CCS angina class from baseline (n = 28 at 3 months** *(white bars)*; **n = 27 at 12 months** *(black bars)*.

and 12 months. Major adverse cardiac events are defined as death, myocardial infarction (Q- and non–Q-wave), and revascularization. Ophthalmologic exams were performed at baseline, 3, and 12 months. Transthoracic echocardiograms were performed at baseline, postoperatively before hospital discharge, and at 1, 3, and 12 months. ETT using either the Bruce (n = 13) or modified Bruce (n = 16) protocols was performed at baseline (within 2 weeks of gene transfer) and then at 1, 2, 3, 8, and 12 months after gene transfer. The initial protocol (Bruce or modified Bruce) was left at the discretion of the investigator and the same protocol used at baseline for each patient was used for the patient's subsequent ETT.

Data are reported as mean ± SD. Comparisons between paired variables were performed using paired, 2-tailed, Student's *t* test with a significance level of $p < 0.05$. One patient died immediately after treatment and is excluded from end point analysis. To assess the functional dependence of the outcome variables on baseline characteristics, logistic regression was used to fit a model to binary response data. Multiple regression was used to fit continuous outcome variables. The 2 regression procedures were performed using Prophet (BBN Systems, Cambridge, Massachusetts). Contingency analyses were performed with either the chi-square test for independence or Fisher's exact test where appropriate.[6]

The average age of the 30 patients enrolled was 59 years (range 39 to 77); 24 patients (80%) were men. Twenty-five patients (83%) underwent prior coronary artery bypass grafting, 13 (43%) underwent previous percutaneous coronary intervention, 19 (63%) had at least 1 prior myocardial infarction, and 10 (33%) had ≥1 myocardial infarction. Average ejection fraction was 50 ± 10%. Risk factors for coronary artery disease included family history of premature coronary artery disease in 21 patients (70%), diabetes in 10 (33%), hypertension in 22 (73%), dyslipidemia in 29 (97%), and history of tobacco use in 26 (87%). CCS class IV angina was present in 18 patients (57%).

Although there were no complications directly related to gene expression, there were periprocedural complications and adverse cardiac events during follow-up (Table 1).There were no perioperative complications such as arrhythmias, congestive heart failure, or hypotension (which has been reported with intracoronary injection of recombinant VEGF protein.[7] In addition to clinical events listed in Table 1, a patient was noted to have an asymptomatic single dot hemorrhage during an ophthalmologic exam 12 weeks after the procedure, and there has been no further progression or clinical sequelae. There were no new malignancies or proteinuria during the 1-year follow-up period.

The average number of angina episodes per week and average number of nitroglycerin tablets used per week significantly improved at all measured time points after gene transfer. Improvement was noted as early as 2 weeks, at which point the average number of episodes decreased from 32 ± 26 to 15 ± 28 and the average number of nitroglycerin tablets used per week decreased from 20 ± 18 to 8 ± 12 ($p < 0.05$ compared

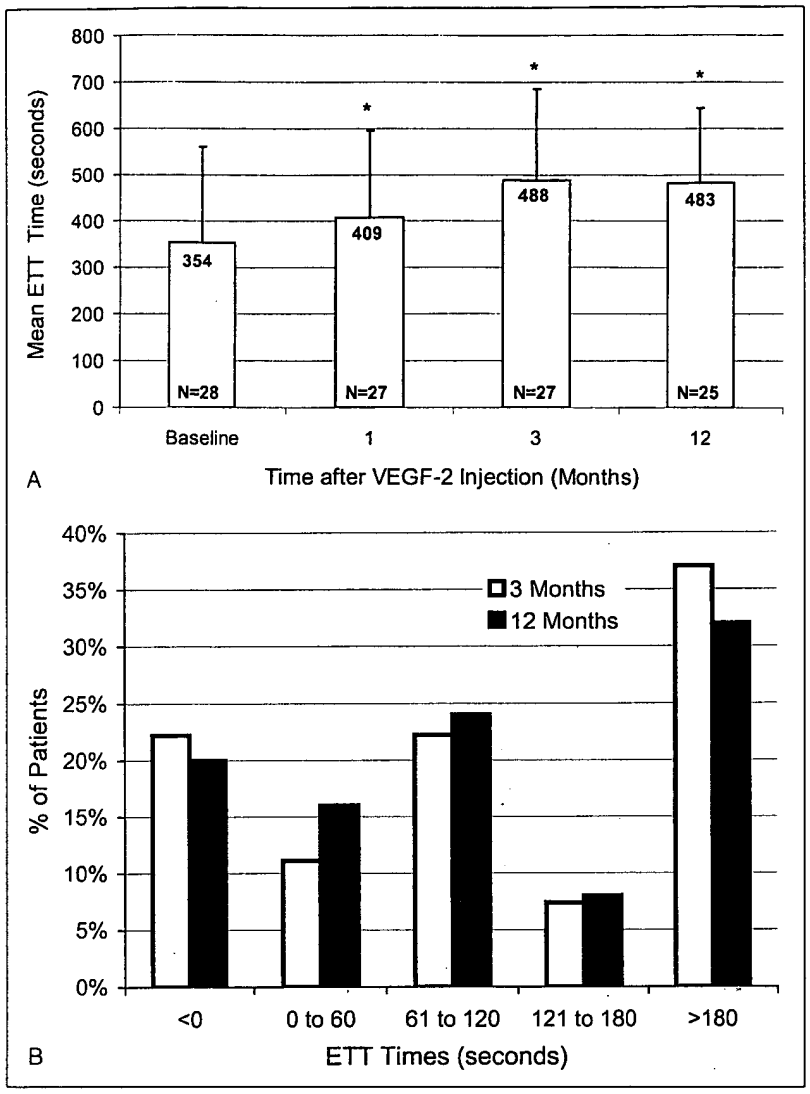

FIGURE 2. *(A)* **Effect of VEGF-2 on mean ETT times. Changes in treadmill times at 3 months compared with baseline in the 3 patients without 12 months follow-up were −96, 471, and 90 seconds. *p <0.05.** *(B)* **Magnitude of treadmill time improvement. Patients are grouped according to improvement (a negative number indicates worsening performance) in ETT times from baseline, baseline versus 3 months (*white bars*, n = 27), and baseline versus 12 months (*black bars*, n = 25).**

with baseline for both angina episodes and nitroglycerin tablets). The maximal treatment effect was noted 3 months after gene transfer when the average number of episodes had decreased to 6 ± 8 and the mean number of nitroglycerin tablets used per week decreased to 2 ± 4 ($p < 0.05$ compared with baseline for both). This observation persisted at 12 months follow-up, when the average number of anginal episodes was 10 ± 19 and average weekly nitroglycerin tablet usage was 3 ± 8 ($p < 0.05$ vs baseline for both).

A statistically significant improvement in CCS angina class was noted at 2 weeks and persisted through 12 months (Figure 1). At 3 months, all patients improved, and at 12 months, 6 patients (22%) improved by 1 class, 20 (74%) improved by at least 2 classes, 7 (26%) were completely free from angina, and only 1 patient had not improved (Figure 1).

ETTs were performed on 29 patients at baseline. The baseline ETT was not obtained in 1 patient. This patient's treadmill time decreased from 174 seconds at 1 month to 157 seconds at 12 months. Both this patient and the patient that died are excluded from analysis. At 1 month, mean treadmill time increased from 354 ± 206 to 409 ± 187 seconds (55 seconds, $p < 0.05$; Figure 2), and further improvement was noted at 3 months when mean treadmill time increased to 488 ± 199 (134 seconds, $p < 0.05$). Twelve months of comparison data are available for 25 patients; average treadmill time improved from 367 ± 213 to 483 ± 162 seconds (116 seconds, $p < 0.05$). Most treadmill times were improved at 3 and 12 months and 1/3 increased by >3 minutes (Figure 2).

There did not appear to be a dose effect because patients improved to a similar degree in all dosage groups. Furthermore, logistic and multiple regression analyses failed to show a correlation between dose and any of these end points: change in anginal episodes, nitroglycerin tablet use, CCS angina class, or treadmill times.

• • •

The present investigation summarizes, for the first time, long-term safety data and clinical outcomes from the first patient population treated with direct, transthoracic, intramyocardial injection of VEGF-2. Direct injection of VEGF-2 deoxyribonucleic acid by way of thoracotomy was well tolerated with no intraoperative complications. Potential adverse events such as arrhythmia, hypotension, or congestive heart failure did not occur. Given the severity of disease in this inoperable, end-stage patient population with class III and IV angina, it is not surprising that 2 patients had perioperative ischemic complications (1 death and 1 myocardial infarction) early in the study. These patients had daily angina at rest before treatment despite multiple medications and became unstable in the immediate postoperative period, perhaps due to obligatory withdrawal of oral antianginal medication during this critical time. It is less likely that these episodes of unstable angina were related to gene expression because, based on data from VEGF-1, expression probably does not peak

sooner than 2 weeks.[8] However, we cannot be certain of this conclusion because plasma levels of VEGF-2 during the present investigation are not available.

One-year mortality and morbidity were low, with 1 death (3%) and 2 myocardial infarctions (7%). Although this study was not designed to assess mortality, this rate compares favorably with published studies of similar no-option patients treated with transmyocardial revascularization showing 1-year mortalities of 5%,[9] 15%,[10] 11%,[11] 12%,[12] and 5%.[13] Myocardial infarction rates at 1 year in these transmyocardial laser revascularization studies ranged from 6% to 15%. In a more benign patient population of "operable" class II and III patients and using an intracoronary injection of an adenovirus vector containing the FGF4 gene, the Angiogenic Gene Therapy (AGENT) trial[14] reported no deaths.

At 1-year follow up, there was minimal evidence of progressive disease elsewhere in the coronary circulation of these patients; only 1 patient required angioplasty to a remote part of the heart. However, longer follow-up will be required to document the progression of disease and whether there is a relation to treatment with VEGF-2 in this patient population with aggressive atherosclerosis.

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Analysis of Baseline Factors Associated With Reduction in Chest Pain in Patients With Angina Pectoris Treated by Enhanced External Counterpulsation

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Data from the International Enhanced External Counterpulsation (EECP) Patient Registry were analyzed to determine which patient characteristics influence improvement in angina class with EECP treatment. Patients with severely disabling angina at baseline, men, and those without a history of smoking are more likely to improve their angina class after EECP, whereas those with diabetes mellitus, prior bypass surgery, and heart failure were less likely to benefit. ©2003 by Excerpta Medica, Inc.

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Enhanced external counterpulsation (EECP) is a noninvasive medical device for treating patients with coronary disease. Three pairs of pneumatic cuffs are applied to the lower extremities and inflated and deflated in synchrony with the cardiac cycle. The cuffs are sequentially inflated (applying 250 to 300 mm Hg of external pressure) at the onset of ventricular diastole, returning blood in the lower extremities to the central circulation, producing aortic diastolic augmen-

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